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Cytoplasmic Fractionation of Kidney Proteins.* (22315)

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The kidney, like the liver, the heart, and certain other organs, has the capacity to hypertrophy and to atrophy under suitable experimental conditions. When one kidney is removed from the rat, the remaining kidney immediately enlarges, and at the end of 6 days, its protein content has increased by approximately 35%(1). If the rat is starved for a period of 7 days, the kidneys may loose 20% of their structural protein(2). It is not known whether the protein added to or lost from the kidney involves certain cellular structures or whether proteins from all cellular components are equally labile. In order to provide an answer to these questions, the distribution of total protein in the various cytoplasmic fractions of normal, hypertrophied, and atrophied kidneys are determined after homogenization and differential centrifugation.

Methods. Female albino rats of the Slonaker-Addis strain weighing 175-185 g were used in all experiments. Renal hypertrophy was produced by removing right kidney while animal was under ether anesthesia and allow-

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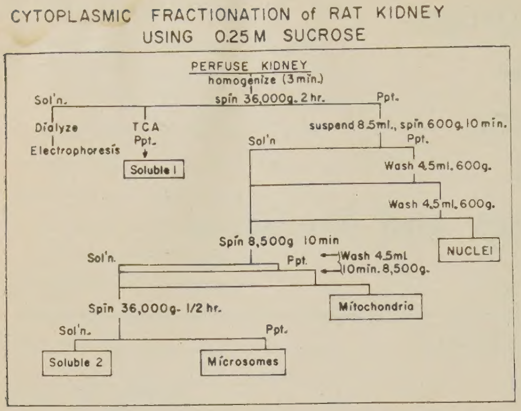

FIG. 1. Flow diagram of fractionation procedure.

ing remaining left kidney to enlarge for 6 days. Renal atrophy was produced by withdrawing all food for 7 days. During this time, the animals subsisted on water only. Rats were anesthetized with ether and their kidneys perfused *in situ* with 0.89% sodium chloride solution as described previously(3). The capsule was removed and the kidneys were blotted dry and transferred to cold room (2°C.) All subsequent steps were carried out at this temperature. Homogenization was carried out in a solution of 0.25 M sucrose containing 0.2% Versene. The homogenate usually contained approximately 3 g of kidney suspended in equal volume of sucrose-Versene solution. The centrifugal method of Hogeboom, Schneider and Pallade(4) was modified slightly as shown in Fig. 1. The particulate fractions were resuspended in distilled water using a pestle designed to fit into the plastic centrifuge tubes. Protein from each fraction was precipitated with 100 ml of 7% trichloracetic acid, washed once with fresh 7% trichloracetic acid, twice with a mixture of equal parts of acetone and ether, once with acetone, and finally once with anhydrous ether. After final centrifugation, the last traces of ether were removed by placing the tubes first in warm water bath and then in oven at 90°C for one hour. The tared tubes containing washed, dried protein were then weighed.

Electrophoresis Procedure. An aliquot of the Soluble 1 fraction was dialysed against boric acid buffer, pH 8.6, for 20 hours at 2°C. Electrophoresis was performed on paper between silicone coated glass plates(5). The paper strips were dried under infra-red lamps and stained with brom phenol blue. The stained paper was cut into strips 0.5 cm wide. The color from each strip was extracted in a solution of 10 parts of concentrated NH_4OH and 90 parts of acetone. Color density of extraction fluid was determined in a Coleman Jr. spectrophotometer at wave length of 610 mμ. The diagrams as shown in Fig. 2 were obtained by plotting per cent absorption on ordinate against distance of migration in centimeters along abscissa.

Results. The results are given in Table I. The data are expressed as means ± standard deviation from the mean. The significance of the differences observed was determined by the method of Fisher(6) for small numbers of observation. P values of <0.01 are taken to indicate a significant difference between the means compared.

It can be seen that the per cent distribution of the total kidney protein in various cytoplasmic fractions studied in normal rats and in starved rats are not significantly different. After 6 days of hypertrophy, the nuclear fraction is slightly, but significantly less, while the mitochondrial fraction is somewhat in-

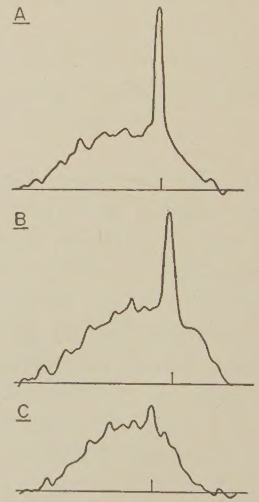

FIG. 2. Electrophoresis diagrams of Soluble 1 fractions. Mobility to the left is toward cathode, mobility to right is toward anode. Arrow indicates point of origin. (A) Normal kidney. (B) Kidney after 6 days hypertrophy. (C) Normal kidney with top fatty layer eliminated.

TABLE I. Percent of Total Protein in Rat Kidney.

Exp. cond.	Control	Neph. 6 days	Starv. 7 days	P value
No. exp.	22	8	8	
Nuclei	19.3 ± 2.7*	14.9 ± 3.3	20.7 ± 1.5	.01 to .001
Mitochondria	21.5 ± 1.5	23.8 ± 2.3	21.3 ± 1.4	.01 to .001
Microsomes	15.8 ± 1.3	15.5 ± 1.4	15.1 ± 0.6	
Soluble 1	27.8 ± 1.4	28.6 ± 1.4	25.5 ± 0.5	
” 2	16.0 ± 1.6	17.1 ± 2.0	17.5 ± 0.8	
” 1 + 2	43.8 ± 2.0	45.7 ± 2.4	43.0 ± 0.8	

* Stand. dev. = $\pm\sqrt{\frac{\Sigma d^2}{n-1}}$.

creased over what is found in kidneys of normal control rats.

Proteins in Soluble 1 fraction of normal and hypertrophied kidneys were subjected to filter paper electrophoresis as described above. Representative patterns are shown in Fig. 2. When the Soluble 1 fraction was withdrawn with a pipette from below a thin fatty surface layer, most of the peak seen at the origin could be eliminated (Fig. 2C.)

Approximately 14 separate peaks can be identified in the patterns from both normal and hypertrophied kidneys. It has not been possible to distinguish electrophoresis patterns of the Soluble 1 fraction as coming from normal kidneys or from hypertrophied kidneys. Differences exist, but they are small and inconsistent.

Discussion. We have been unable to find reports in the literature dealing with this type of analysis of kidney or other visceral organs undergoing hypertrophy or atrophy. While the flux of tissue protein of liver and kidney during regeneration and atrophy may not be strictly analogous, it will be pertinent to refer to certain experiments performed on liver proteins under these circumstances. Thus, Soroff, Claus and Cohen(7) studied proteins of rat liver by the technic of free electrophoresis. They state that throughout an 8 day period of liver regeneration following 70% partial hepatectomy and throughout a 9 day fast, electrophoretic analysis of soluble rat liver proteins showed no significant difference from those of normal rat liver extracts. de Lamirande, Allard and Cantero(8) report that no difference exists in the soluble proteins of cell fractions from regenerating and normal liver by the technic of electrophoresis. However, a decrease in certain components of the mitochondrial fractions of regenerating liver was observed. Luck(9) found that various fractions of liver protein increase equiproportionally with increased protein intake.

It is clear from the present study that from a loss of 20% of the kidney tissue protein during a fasting period through a gain of approximately 35% during a period of compensatory hypertrophy, the relative protein content of the various cytoplasmic fractions of the kidney remains essentially unchanged. This would indicate that under the experimental conditions outlined above, protein is lost from or added to all cellular fractions of the kidney in a nearly equiproportional manner. The results are inconsistent with the view that a certain fraction of the cellular protein of the kidney is more labile than other fractions under conditions of atrophy and hypertrophy.

Summary. 1. Distribution of kidney proteins in various cytoplasmic fractions has been determined in normal, hypertrophied, and atrophied rat kidney. The Soluble I fraction from normal and hypertrophied kidneys has been subjected to paper electrophoresis. 2. Despite a gain in total protein of approximately 35% in the hypertrophied kidney, and a loss of approximately 20% in total protein in the atrophied kidney, the distribution of proteins in the various cytoplasmic fractions shows only minor deviations from the normal. 3. No consistent differences could be seen in electrophoresis patterns of the Soluble I fraction between normal and hypertrophied kidney. 4. The significance of these findings is discussed.

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Structures Responsible for the Characteristic Purple Color Development in Diphenylamine Reaction with Serum.* (22316)

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Numerous workers(1-4) have reported that the intensity of purple color which resulted when serum was heated with Dische reagent (5) was increased in a variety of diseases. The reaction has been associated with the serum proteins containing carbohydrate moieties: mucoproteins or glycoproteins found in the alpha globulin fraction of serum(2,6,7). Mucoproteins isolated from bovine tonsils and from human urine also gave the color reaction (8,9). The color reaction has been attributed to the presence of sialic acid(10) in the mucoprotein molecule(7). A number of polyhydroxylic acids, neuraminic(11,12), hemataminic(13), and lactaminic(14), have been isolated and appear to be related to sialic acid(15).

It is the purpose of this report to point out that the structure for sialic acid proposed by Gottschalk(16,20) is consistent with structures known to give the characteristic color development when heated with the Dische reagent.

Absorption Studies. Purple color which results when serum or mucoproteins are heated with the reagent is due to 2 absorption regions in the visible spectrum (Fig. 1). One absorption maximum occurs at about 520 mμ while the second occurs at 650 mμ. The absorption maximum at about 520 mμ is the significant one. The second absorbing region at 650 mμ can be attributed to conversion of common hexoses present in mucoproteins to 5-hydroxymethylfurfural when heated in an acid solution(17,18). Absorption maxima in the vicinity of 520 mμ, as will be shown below, can probably be attributed to a deoxyhexose struc-

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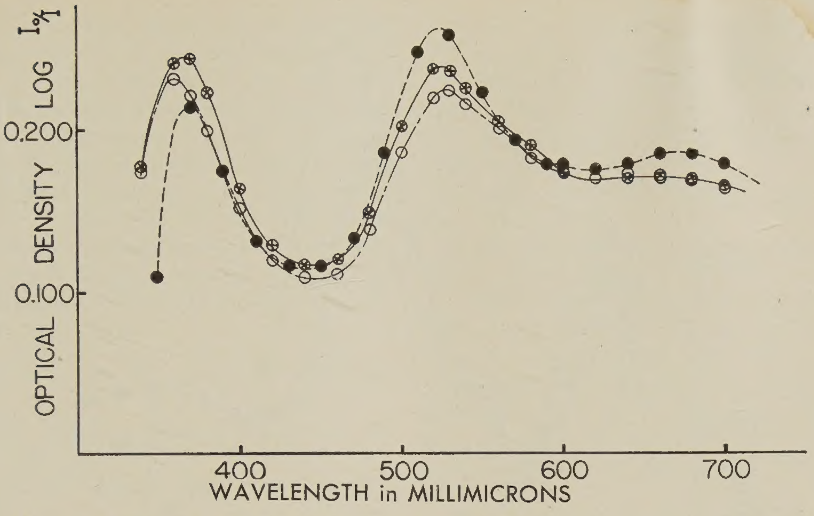

FIG. 1. Absorption curves from diphenylamine reaction obtained with human serum, sialic acid and bovine tonsil mucoprotein. × (circled) Human serum 0.12 ml serum/ml solution according to published procedure(2) using Dische reagent. ○ Bovine tonsil mucoprotein 590 μg protein per ml solution. ● Sialic acid 130 μg/ml solution. To 1 ml solution was added 2 ml of reagent. The mixture heated for 30 min. at 100°, cooled to room temperature and read in a Beckman Model DU Spectrophotometer.

ture which has been postulated(16,20) to be present in sialic and neuraminic acids.

The absorption curves obtained when 40 μg of 2-deoxygalactose[§], 40 μg of D-galactal, and 10 μg of fructose, were heated with Dische's diphenylamine reagent may be seen in Fig. 2. In the case of 2-deoxygalactose, and D-galactal, the 40 μg samples were dissolved in 1 ml of water. Two ml of the reagent were added to the aqueous solution and the mixture was heated for 30 minutes in a boiling water bath. Fructose treated in this manner did not develop the characteristic color. When the Dische reagent was added directly to as little as 10 μg of fructose, however, in the absence of water, and the mixture was heated, a pink color developed within a few minutes.[‖] The absorption curve obtained with dry fructose heated with the Dische reagent closely resembled the curves obtained from D-galactal and 2-Deoxygalactose as can be seen in Fig. 2. Many substances have been tested with the Dische reagent both in our laboratory and elsewhere(18). Of all the simple substances tested, only the deoxyhexoses and hexals gave the characteristic color development and absorption behavior, when heated in aqueous solution with the unmodified Dische reagent. The ketoses in aqueous solution, when heated with a modified reagent containing a larger fraction of sulfuric acid, also give the characteristic color development. The absorption curves are similar in this case to those obtained when serum, mucoproteins, the hexals and the deoxyhexoses are heated with the Dische reagent.

The absorption curves obtained when sialic acid and a purified mucoprotein were heated with Dische reagent are shown in Fig. 1. Comparison of curves in Fig. 1 and 2 indicate a striking similarity in chromogenic structures present in all substances reacting with the reagent. Acid-catalyzed dehydration of hexoses is well known(19). The conclusion that water is removed from 2-deoxyhexoses and from ketoses under the conditions discussed above, seems justified. The ethylenic ring seen in Fig. 3 thereby becomes a feature common to a dehydrated deoxyhexose, dehydrated fructopyranose, and to hexals. This structure or a common derivative of this

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[§] 2-deoxyglucose also shows this characteristic behavior.

[‖] Sorbose, galaheptulose, mannoheptulose and sucrose also behave in similar manner.

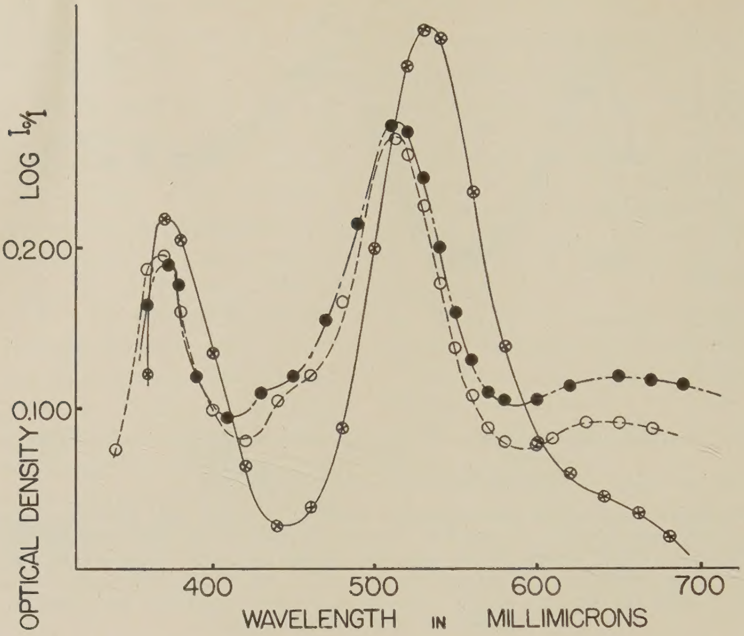

FIG. 2. Absorption curves from diphenylamine reaction obtained with 2-deoxygalactose, D-galactal and fructose. ○ 2-deoxygalactose 40 μg/ml solution. ● D-galactal 40 μg/ml solution. × (circled) Fructose 10 μg. To 1 ml solution was added 2 ml of reagent. The mixture was heated 30 min. at 100°, cooled to room temperature and read in a Beckman Model DU Spectrophotometer. In the case of fructose 3 ml of reagent was added to 10 μg of dry fructose and the mixture heated 30 min. at 100°.

structure accounts for the absorption maxima at approximately 520 mμ. A deoxyketose structure in sialic acid(16,20) would therefore account for the color reaction of this substance with the Dische reagent.

The chemistry of the deoxyhexoses(21) and the hexals(22,23) is also consistent with the viewpoint that a deoxyhexose is present in the sialic acid molecule. A characteristic of the deoxy sugars is their great reactivity and their instability in solutions containing mineral acids. 2-deoxygalactose is almost completely destroyed, with marked discoloration, when heated with 1 N HCl for one minute at 100°. The glycals are stable in alkaline solution, form humins in acid solution and are extremely reactive substances(22,24). The pronounced tendency of sialic acid(25) and of the gangliosides(26) to form humin when heated with acids has been described.

Summary. It has been shown that deoxyhexoses and hexals react with the Dische diphenylamine reagent in a characteristic manner. No other structures except sialic acid and related compounds have been found to react in the same fashion. A deoxyhexose structure for sialic acid has been proposed by Gottschalk. The color given by sialic acid when heated with the reagent is therefore accounted for by the presence of the deoxyhexose. Since sialic acid is considered to be a common constituent of the glycoproteins (mucoproteins), the color given by these substances and serum when heated with the Dische reagent appears to be due to the deoxyhexose present in sialic acid.

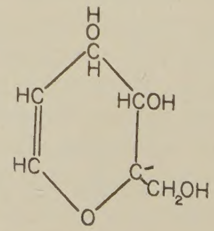

FIG. 3. Ethylenic ring structure common to glycals, and after elimination of a molecule of water, to deoxyhexoses and pyranose form of ketohexoses.

The assistance of Miss Saima Lagg is gratefully acknowledged. Discussions with Dr. G. Blix have been helpful. We are grateful to Dr. A. Gottschalk for calling attention, prior to our seeing the published reports, to the deoxyhexose character of his proposed structures. A number of substances used in this study were obtained through the kindness of several individuals. Dr. T. Reichstein sent generous samples of 2-deoxygalactose, 2-deoxyglucose and D-galactal. The late Dr. G. S. Hudson provided galaheptulose and mannoheptulose. The sample of sialic acid was obtained from Dr. L. Odin.

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Hormonal Requirements of Implantation in the Rabbit.* (22317)

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The phenomenon of implantation in mammals is accomplished by a definite sequence of events which in part are dependent on the endocrine system. It has been adequately demonstrated(1,2,3) that blastocyst growth in the rabbit is controlled by the secretion of the corpus luteum. Partial progestational proliferation in the ovariectomized rabbit could be produced within 5 days by 0.2 mg progesterone daily(4). The injection into rabbits, ovariectomized 20 hours after mating, of progesterone in dosage up to 1.5 mg a day maintained implantation until the 11th day of pregnancy(5). Crude corpus luteum extracts of similar unitage maintained embryos to full term(6). In ovariectomized rabbits, implantation is maintained for 11 days if at least 0.5 mg of progesterone is given twice daily; 1.0 mg given twice daily after the 11th day of pregnancy maintains the embryo to term(7). In the intact non-ovulated rabbit, implantation of transplanted ova requires an initial high dose of progesterone; subsequent

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[†] On leave from Faculty of Agriculture, University of Cairo, Egypt.

treatment prevents the degeneration of the embryo or death of the fetus(8).

Materials and Methods. Sixty-three post-partum and 12 adult virgin rabbits were purchased from Rockland Farms, N. Y. and local dealers and were kept in this laboratory. Two series of experiments were carried out: 1) Eight groups of 3 to 5 animals each were mated twice to fertile bucks and injected intravenously with an ovulating dose of 12 mg of sheep pituitary extract. The does were ovariectomized 24 to 48 hours post coitum and the ovulation points on each ovary were counted. Six groups of does were injected subcutaneously once daily with an oily solution of 0.3, 2 or 25 mg of progesterone 2 or 4 days post coitum. Two other groups were injected subcutaneously once daily with 2 or 25 mg progesterone together with 0.1 mg estradiol. 2) Four groups of 3 does each were mated to a vasectomized buck and injected intravenously with an ovulating dose of sheep pituitary extract. The does were ovariectomized as above; immediately before ovariectomy 24 to 30 fertilized one-day ova were transplanted into the left tubes. The ova were obtained from virgin donors which were superovulated with sheep pituitary extract(9) and mated at the same time as the recipients so that the transplanted ova would be of a corresponding stage with that of the recipient. The experimental does were injected subcutaneously with 2 or 25 mg of progesterone once per day starting either on the day of ovariectomy or the 4th day after mating. In 2 groups 0.01 mg of estradiol was injected once daily. All does were sacrificed on the 10th day after mating; the uteri were examined to determine the number of implantation sites, the diameter of uterine swellings and the weight and length of the fetus. In cases where implantation did not occur, the uteri were flushed with saline and examined under the dissecting microscope. Two control experiments were carried out: (a) Twelve does were mated to fertile bucks, and sacrificed on the 10th day after mating. The number of implantation sites in relation to the number of corpora lutea were counted and the average diameter of uterine swellings, weight and length of the fetus were recorded. (b) Six does were mated to fertile bucks, and were injected subcutaneously once daily with 2 or 25 mg of progesterone. These were sacrificed 15 days after mating and the uteri examined.

Results. In the ovariectomized animals the number of ovulation points on both ovaries ranged from 5 to 18 with an average of 10.9. Implantation occurred in all the animals when the progesterone dose was 2 or 25 mg (Table I). The number of implantations in both uteri ranged from 1 to 3. The number of implantations expressed as a percentage to the total number of ovulation points ranged from 5% to 33% with an over-all average (of 6 experimental groups) of 19.21%. There was no difference in the percentage of implantations between the 2 or the 25 mg groups nor between the groups whose initial injection was on the 2nd or 4th day after mating. Im-

TABLE I. Effect of Progesterone on Implantation and Development in Ovariectomized Rabbits.

Hormones inj. (mg/day)	Start of inj. post coitum (days)	No. of does	Avg No. of ovulations in both ovaries	% of implantations		% of viable embryos		% of ova (or blastocyst) recovered by flushing	
				Range	Mean	Range	Mean	Range	Mean
.3 progesterone	2	3	12.3	0	0			50–73	53
	4	4	7.7	0	0			41–69	52
2 progesterone	2	4	13.3	11–21	19.00	33–50	38		
	4	3	11.3	15–20	17.66	33–66	50		
25 progesterone	2	5	10.4	12–33	23.33	20–80	35		
	4	4	10.2	9–20	15.33	33–66	47		
2 progesterone + .1 estradiol	2	5	7.2	0	0				
25 progesterone + .1 estradiol	2	5	10.1	0	0				

TABLE II. Effect of Progesterone and Estradiol on Implantation of Transplanted Ova in Ovariectomized Rabbits.

Hormones inj., mg/day P*	E*	Doe No.	Left side after sterile mating No. of ovulation points	No. of transferred eggs	No. of implantations	% of implantations	% of viable embryos
2	—	25	3	27	3	11	33
		26	4	24	4	17	50
		27	5	28	2	7	50
2	.01	28	6	25	3	12	66
		29	5	30	2	7	50
		30	5	30	2	7	50
25	—	31	6	30	2	7	50
		32	7	27	2	7	50
		33	4	27	3	11	100
25	.01	34	5	30	1	3	100
		35	3	30	2	7	50
		36	5	29	2	7	50

* P = Progesterone; E = Estradiol.

plantation failed to occur at a dose of .3 mg progesterone, 2 or 25 mg progesterone in combination with 0.1 mg estradiol. In the group injected with 0.3 mg progesterone daily, 3 to 7 ova (or blastocysts) were recovered from the uterus. In the groups injected with 0.1 mg estradiol with progesterone, no ova (or blastocysts) were recovered from both uteri. A few degenerating ova were recovered from the tubes of 3 animals out of 10.

In the control groups "a" which were mated and sacrificed at 10 days the number of implantations expressed as a percentage of the total number of ovulation points ranged from 71 to 100 with an average of 81 and the percentage of viable embryos ranged from 70 to 100 with an average of 81. Judging by the diameter of the uterine swellings and the developmental stage of the embryos in the ovariectomized animals compared with that in the intact animals, there was no evidence of delayed implantation in the former group.

When the gravid uteri of the ovariectomized animals were dissected 35 to 50% of the embryos were viable. In the control group "b" which were injected with 2 mg of progesterone, the percentage of implantations ranged from 80 to 100 with an average of 93 and the percentage of viable embryos ranged from 67 to 100 with an average of 79. In the control group injected with 25 mg progesterone, the percentage of implantations ranged from 80 to 90 with an average of 82. There were no significant differences between these 2 control groups "b". Also the injection of 2 or 25 mg of progesterone in the intact animals did not exert an effect on the percentage of implantation.

In the second series of experiments where fertilized ova were transplanted in the left horn of ovariectomized animals, the number of implantation sites ranged from 1 to 4 and the percentage of implantations ranged from 3 to 17 (Table II). It was noted that the number of implantations was not corresponding to the number of transferred ova. In fact the number of implantations in the one horn was equivalent to those of 2 horn in the previous experiment.

Discussion. There is general agreement that the presence of the luteal hormone is essential for successful implantation but the mechanism by which it makes implantation possible is not fully known. Results of the present experiment suggest that the hormonal requirement for embryonic development is not identical to that required for implantation. The 2 days following implantation seem to be the most critical during the embryonic life (10). It is possible that substances other than progesterone such as androgens or estrogens are required in certain forms or quantities to ensure normal development. The dose of 2 or 25 mg of exogenous progesterone in the ovariectomized rabbits is sufficient to establish implantation but not for further development in some of the cases. Courrier and Kehl (11) and Allen and Heckel(12) have determined that while in the early stages about 1 mg of progesterone is sufficient, in later stages up to 5 mg daily is necessary. Meanwhile ovarian hormones are completely unnecessary in experimental extrauterine pregnancy(13,14).

The absence of implantation when 0.3 mg progesterone was injected daily is either due to incomplete progestational conditions of the endometrium or to a delay in the transportation and reception of ova from the tubes to the uterus. It was thought that the injection of a low dose of progesterone may result in delayed implantation. Chang(8) has indicated that the injection of a low dose of progesterone in non-ovulated rabbits for 5 to 6 days, only renders the endometrium similar to that of a rabbit in the 3rd to 4th day of pseudopregnancy. Delayed implantation occurs spontaneously in mice as a result of lactation(15) or may be induced by the administration of ovarian hormones(16). It is possible that implantation was not delayed in the present investigation due to the fact that progesterone is rapidly destroyed (or converted into an inactive form)(17,18), particularly in the ovariectomized rabbit(19). Courrier and Kehl(11) have shown that a definite progesterone:estrogen ratio of 750:1 is required for successful implantation in the ovariectomized rabbit. In the present experiment when the progesterone:estradiol ratio was narrow (250:1 or 20:1), the ova were blocked in the tube and degenerated before reaching the uterus.

It is of great interest to note that the actual number of implantations in the two series of the present experiment did not increase with the increased number of ova in the uterus. This may denote a quantitative basis for the hormonal requirement of implantation. Further experiments are needed to explain the high frequency of embryonic mortality in ovariectomized progesterone-treated rabbits. The role of placental hormones in such animals should not be overlooked.

Summary. The role of ovarian hormones in implantation was studied in rabbits ovariectomized after mating and in ovariectomized pseudopregnant rabbits into which fertilized ova were transplanted. There was no evidence of delayed implantation. One to 3 implantations occurred in ovariectomized rabbits injected with 2 or 25 mg progesterone. The percentage of implantations ranged from 15 to 23 irrespective of dosage or the time at which injection started. Implantation failed to occur when the dose was 0.3 mg progesterone or 0.1 mg estradiol in combination with progesterone. The number of implantations did not increase with the transplantation of ova. This may suggest a quantitative basis for the hormonal requirement at implantation. Hormonal requirements at implantation vary from those needed for subsequent development.

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Evaluation of the Sakaguchi Reaction for Quantitative Determination of Arginine.* (22318)

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Sakaguchi(1) reported a color reaction specific for arginine which several investigators have since attempted to adapt for quantitative use. Weber(2) suggested that under certain conditions the reaction could be modified to give a quantitative colorimetric method. The procedure of Weber was further modified by Jorpes and Thoren(3) and Fisher and Wilhelmi(4), who attempted to reduce the rapid fading of color noted by Weber. MacPherson (5,6) attempted to eliminate color fading by adding urea prior to the development of color and by using a high concentration of chromogenic reagent (α-naphthol) and developing reagent (potassium hypobromite). In our hands, this procedure gave a highly colored blank with no improvement in color stability. Sakaguchi(7) reported that if 8-hydroxyquinoline was used in place of α-naphthol, a more stable color was produced. However, we have been able to detect little difference in the stability of colors formed with either chromogenic agent.

We have evaluated the published methods and modifications of these methods and have derived a procedure for estimating arginine which is highly satisfactory from the points of view of sensitivity and duplicability. Levels of arginine as low as 2 γ can be determined quite accurately.

Experimental. Reagents. Arginine hydrochloride—10 γ arginine/ml; prepared as needed from stock solution containing 605 γ arginine hydrochloride/ml, kept at 5°. This solution is stable for about 1 month. *Sodium hydroxide*—10% solution in water. *8-Hydroxyquinoline*—0.02%; prepared by diluting a 0.2% solution in ethanol with distilled water. *Sodium hypobromite*—1%; prepared by diluting 1 g liquid bromine to 100 ml with 5% sodium hydroxide. This is stable for 1 month in the cold and dark. *Urea*—40% solution in water. *Procedure*—All reagents and solutions should be cooled in ice bath before beginning the determination. To a tube containing 5 ml of the test solution (0—30 γ arginine) are added 1 ml of 0.02% 8-hydroxyquinoline and 1 ml of 10% sodium hydroxide. The solutions are mixed thoroughly and replaced in the ice bath for 2 minutes. Then 0.2 ml of 1% sodium hypobromite is added rapidly to develop the color. After mixing and within 15 seconds, 1 ml of 40% urea is added to destroy excess hypobromite and prevent rapid fading of color. One minute after addition of the hypobromite, 5 ml of cold distilled water are added, the solutions mixed, and the absorption is read at 490-510 mμ. It is important that the sample be read within 5 minutes after development of color. Also the readings should be made at the same time interval after color development in each set of analyses.

Standard Curve. A set of 4 tubes is prepared containing 0, 10, 20, and 30 γ of arginine. The blank is developed first and is used to bring the colorimeter or spectrophotometer to zero. The other tubes are then developed in the order given above and results read and plotted.

Results. A linear relationship exists between optical density of the color developed and concentration of arginine in the range of 0-50 γ of arginine per tube; however, it is recommended that 30 γ be the upper limit when using ordinary colorimeters.

Fig. 1 shows a typical standard curve prepared as described above. This curve is reproducible from one experiment to the next within ±0.5%. In Fig. 2 is presented the

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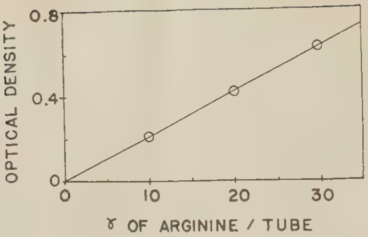

FIG. 1. Typical standard curve for arginine.

absorption spectrum of the color produced in the reaction. It can be seen that the maximum absorption occurs near 506 mμ. Reasonably good quantitative results can be obtained in the range 490-510 mμ, however.

Sakaguchi(1,7) and Poller(8) have studied the nature of compounds which interfere with this color reaction. Glycine appears to give the most interference among the amino acids. Glycine was found to cause a slight color intensification when present in small amounts (up to 100 γ/developing tube) in a solution with arginine. When the concentration of glycine in the developing tube reaches 150 γ or above, however, color fading is noted. In either case if a standard curve is established with a specific amount of glycine, arginine can still be determined.

Our work indicates that arginine may be determined in the presence of mixtures of amino acids. Those we have investigated were amino acid mixtures similar to casein and egg albumin hydrolysates(9). Thus, recovery experiments using amino acid mixtures containing known amounts of arginine indicate that accurate results are easily obtained.

In this laboratory a number of buffers commonly used in biochemical studies (tris(hydroxymethyl)aminomethane, glycine, acetate, phosphate, pyrophosphate, and bicarbonate) were also investigated for possible interference. Only glycine and tris(hydroxymethyl)-aminomethane ("tris") were found to interfere to a measurable extent. It should also be noted that the level of sodium hypobromite is quite critical since an excess tends to cause decomposition of the color produced. Of the series of hypobromite concentration studied, 0.2 ml of 1% sodium hypobromite was found to give full color development and negligible color fading if read within 5 minutes.

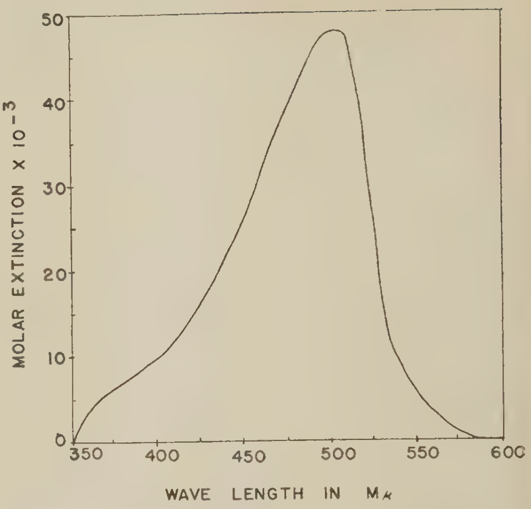

FIG. 2. Absorption spectrum of the colored product formed with arginine.

Summary. The Sakaguchi reaction has been studied to obtain a reliable quantitative method for the colorimetric estimation of arginine. The best conditions for this method have been investigated and reported. The method has been found to be entirely satisfactory for determining arginine over the range of 0-30 γ. Duplicability of the method is ± 0.5%, (Standard deviation).

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Colorimetric Method for Rapid Determination of Arginase Activity.* (22319)

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Existing methods for determining arginase activity generally involve measuring one of the products of arginine hydrolysis. For example, ornithine has been determined by titration(1). The most usual product measured, however, is urea, which can be estimated by the cumbersome procedure of decomposition with urease followed by the manometric measurement of carbon dioxide(2,3) or the colorimetric(4) and titrimetric(5) estimation of the ammonia produced. Urea can be directly estimated colorimetrically, but arginine has been reported to interfere with the color produced(6). In addition, most of these methods require more arginase activity than many studies allow. We recently had need of a rapid method for arginase determination in which only small amounts of tissues were required. A note by Iyer and Krishna Murti (7) appeared to offer a promising method, in which arginine remaining after hydrolysis was estimated colorimetrically by the Sakaguchi reaction as modified by MacPherson (8). However, the method of Iyer and Krishna Murti(7) could not be reproduced since the details of carrying out the method as well as the results presented could not be adequately interpreted. It was also found that the suggested glycine buffer gave marked inhibition of color development.

In the present paper, we wish to present a method which allows the accurate determination of arginase activity in samples of liver as small as 20 γ (fresh weight).

Methods. Reagents. In addition to those reagents listed by Gilboe and Williams for determining free arginine(9), the following reagents are required for estimating arginase activity: 0.4% trichloroacetic acid (TCA); 0.1 M sodium pyrophosphate adjusted to pH 9.5; and a solution containing 50 γ of arginine per ml, prepared as needed from a stock solution containing 605 γ arginine hydrochloride per ml kept at 5°. The last solution is stable for about one month. *Procedure.* A liver homogenate containing 0.1 mg of liver per ml is prepared by homogenizing a weighed portion of rat liver, obtained from a freshly sacrificed rat, in ice-cold distilled water. The homogenate should be kept in an ice bath and used within 30 minutes after preparation. In a test tube, large enough for mixing of added solutions by swirling, are placed 3 ml of the diluted arginine solution and 1 ml of the pyrophosphate buffer. The mixture is placed in a constant temperature bath maintained at 37°. After a 5-minute temperature equilibration period, 1 ml of the liver homogenate is added; the contents are mixed quickly, and the mixture is incubated for exactly 10 minutes. At the end of the 10-minute period, 1 ml of the incubation mixture is withdrawn and pipetted into a tube containing 4 ml of 0.4% TCA. The protein precipitated from this amount of tissue is so low that centrifugation is not necessary. However, if larger amounts of tissue are used, for example, tissues containing exceptionally low arginase activity, centrifuging would be necessary to obtain a clear extract. The procedure for estimating the unhydrolyzed arginine has been outlined by Gilboe and Williams(9). A blank is run using the same procedure as above except that the arginine is omitted. Since each tube except the blank contains 30 γ of arginine per ml before incubation, the amount of arginine hydrolyzed is determined by subtracting the γ of arginine per ml remaining in the tube from 30 γ. By decreasing all volumes to one-fifth those given above and adding 4 ml of TCA directly into the incubation mixture, only 20 γ of liver are needed.

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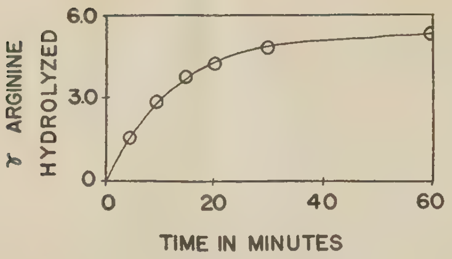

FIG. 1. Rate of arginine hydrolysis in a typical experiment.

A *standard curve* should be established by mixing 0, 1, 2, and 3 ml of the arginine solution (50 γ per ml) in 4 separate tubes, adding 1 ml of 0.1 M sodium pyrophosphate to each tube, diluting to 5 ml with water, and following the procedure outlined above for taking the sample and developing the color. This method of obtaining a standard curve gives both a standard curve and a check on the procedure.

Results. To determine the proper incubation time, a mixture of 6 ml of 50 γ of arginine per ml, 3 ml of the pyrophosphate buffer, 3 ml of distilled water, and 3 ml of the liver homogenate was incubated at 37°. One ml aliquots were taken from the incubation mixture at 5, 10, 15, 30 and 60 minutes. The amount of arginine hydrolyzed was determined as previously described. Fig. 1, which is a plot of the arginine hydrolyzed *vs.* time, indicates there is no longer a straight line relationship between enzyme activity and time after 10 minutes. This observation agrees with the results of Van Slyke and Archibald(6).

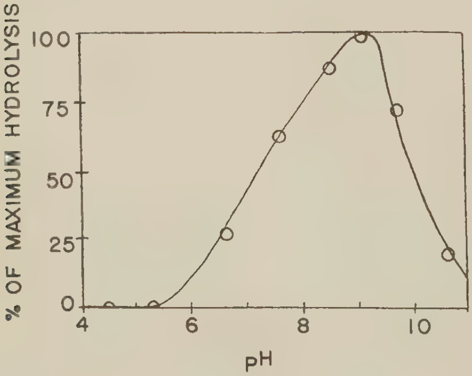

FIG. 2. Effect of pH on arginase activity (avg of 2 experiments).

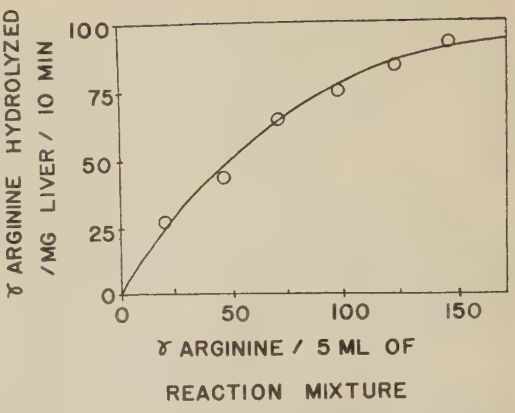

FIG. 3. Arginase activity *vs.* arginine concentration (avg of 7 experiments).

The optimum pH of the enzyme was determined by carrying out the reaction in a compound buffer (0.05 M in each anion) prepared from the following substances: sodium acetate, acetic acid, sodium dihydrogen phosphate, disodium phosphate, sodium pyrophosphate, sodium carbonate, and sodium bicarbonate. Portions of this compound buffer were adjusted to pH 4, 5, 6, 7, 8, 9, 10 and 11 respectively. From Fig. 2 it can be seen that the pH optimum lies between 9.0 and 9.5, which is in agreement with the data of previous workers for arginase(6,10).

The substrate concentration curve for 0.1 mg rat liver per 5 ml total reaction volume is shown in Fig. 3. It can be seen that the curve levels off near 150 γ of arginine. The linearity of the enzyme activity *vs.* enzyme concentration over a 5-fold range using 150 γ arginine is shown in Fig. 4. Thus it may be concluded that the enzyme in 0.1-0.5 mg of liver per 5 ml total reaction volume is saturated when the amount of substrate is 150 γ.

Further experiments have indicated that additional manganese is not needed in the system reported in this paper. This indicates that sufficient manganese is present in the whole liver homogenate to activate the enzyme maximally. Using purified arginase preparations, manganese would undoubtedly need to be added. This is left to the discretion of the operator when preparations other than whole liver homogenates are used. From data in Fig. 3 the Michaelis constant for this

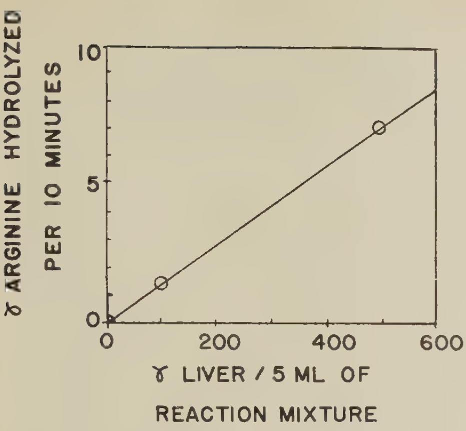

FIG. 4. Linear response of arginase activity *vs.* liver concentration (avg of 2 experiments).

system was calculated to be 1.53 x 10^{-4} M which agrees with a calculation of the Michaelis constant from data presented by Van Slyke and Archibald(6).

Summary. A method has been presented for the rapid determination of arginase with a minimum of operations and equipment. The activity of as low as 20 γ of whole liver can be determined. The arginine remaining after enzymatic hydrolysis is estimated by a quantitative modification(9) of the Sakaguchi reaction. Additional studies are presented indicating the necessary substrate concentration, the pH optimum, and linearity of the enzyme concentration curve for these experimental conditions.

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Effect of Cholinergic Drugs on Development of Chick Embryo.* (22320)

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There is sufficient evidence to indicate the presence of cholinesterase in embryos. Zacks (10) demonstrated the presence of cholinesterase in the embryonic chick from 0-96 hour stages. Wenger(8) followed the development of cholinesterase in spinal cords of chick embryos of 5½ to 11 days of incubation. Nachmansohn(4,5) determined cholinesterase activity in chick nerve and muscle at 6 or 9 days of age. He followed the increasing levels of activity of this enzyme to about the 15th day and showed a higher rate of increase until hatching. Similar observations have been made on early and later stages of amphibian embryos (Youngstrom(9), Sawyer (6,7), Boell and Shen(1,2)).

Since cholinesterase is present during early embryonic stages and increases steadily as the neuromuscular apparatus appears, the administration of enzyme inhibitors and the accumulation of acetylcholine could be attended by developmental abnormalities. To investigate this possibility, 4-day chick embryos were treated with various doses of cholinesterase inhibitors (prostigmine bromide; physostigmine salicylate), the enzyme

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TABLE I. Effects of Acetylcholine (Ac) and Atropine (A) on Development of Chick Embryos.

Drug	Route	Eggs	mg of drug or 0.85 cc NaCl at days of incubation								Mortality				Sac.	Wt (g)	Comments
			4	5	6	7	8	9	11	12	5	6	7	8	13		
Ac	A.s.	2	.5	.25								2					
		2	.5	.25	.5		.4	.4	.4	.4					2	8.5, 7	Viscera ectopic
		1	1.0	.50	.2		.2	.2	.2	.2					1	9	Normal
	Y.s.	2	.3	.3								2					
		2	.25	.25	.3		.25	.25	.25	.25					2	8.6, 9.3	Viscera ectopic
		1	.5	.5							1						
	E.m.	2	.2	.2		.2					1			1			
		1	.2	.2		.2	.2		.2	.2					1	9	Normal
A	Y.s.	1	.1	.1	.1		.1		.1	.1					1	8.9	
		4	.4	.4	.4							2	2				
	E.m.	2	.1	.1							1	1					
		3	.2								3						
		1	.2	.1	.2		.2	.2	.2	.2					1	8	Normal
		3	.5	.5	.1		.1	.1	.1	.1					3	7.5, 8.8, 8.9	”
S	E.m.	2	.2	.2	.2		.2	.2	.2	.2					2	9.8, 10	”
Cn		2													2	12, 12.5	”
Totals		31									6	7	2	1	15		

S, 0.85 saline controls; Cn, untreated embryos; Sac., time of sacrifice in days of incubation; A.s., air sac; Y.s., yolk sac; E.m., on the embryo.

substrate (acetylcholine) and a cholinergic blocking agent (atropine).

Method. Eggs containing vigorous 4-day embryos were obtained from a local hatchery. Various amounts of atropine, acetylcholine, eserine salicylate, and prostigmine bromide, were administered in either the yolk sac, air sac, or directly on the embryo. Since boric acid is used as a preservative in the prostigmine bromide preparation, control embryos were treated with 0.01% boric acid and 0.85% sodium chloride. Animals were sacrificed at 7-13 days of incubation, fixed in Bouin's or DeCastro's fluid and stained with Cajal silver and hematoxylin technics. Preparations with silver were used to examine the nervous system.

Results. Large amounts of acetylcholine (1.20 mg-2.85 mg) injected during the interval of 4-12 days of incubation were well tolerated as were similarly administered large doses of atropine (0.60 mg-1.50 mg). Of 27 embryos treated, 11 survived 13 days' incubation, and except for slight decreases in weight and ectopic viscera all were grossly of normal appearance (Table I).

During the interval of 4-8 days' incubation 42 embryos were treated with physostigmine bromide, eserine salicylate and a combination of physostigmine bromide and acetylcholine (Table II). The total amount of eserine or physostigmine given to each embryo was within the range of 1.0 mg-15.0 mg. Of the above 42 embryos, 22 survived 7-13 days' incubation and in all but 3 of the survivors, a remarkable kyphosis syndrome was observed (Fig. 1-5).

The syndrome as observed in 9-13-day embryos is briefly described (Fig. 1-5). The head was approximately the same size as controls, eyelids were retarded, the lower beak shortened, and the external auditory meatus connected with a fissure extending inferiorly and anteriorly. The meatus was larger than in controls. The neck and trunk were reduced approximately one half in length and this can be accounted for by 3 sharp compensating S-shaped curvatures in the cervical, thoraco-lumbar, and sacral-coccygeal vertebrae, respectively (Fig. 5). Bodies of the vertebrae were compressed and wedge-shaped at levels of sharp bending. Ankylosis of vertebrae was observed at levels where the vertebrae were compressed. Upper and lower extremities were shortened.

Throughout all levels of the spinal cord of

TABLE II. Injection of Prostigmine Bromide (P) and Eserine Salicylate (E) into Yolk Sac of Embryonic Chick and Effects on Development.

		Days of incubation																		
		Drug (mg) or 0.85% NaCl (cc)					Mortality						Sacrificed						Sn	
Drug	Eggs	4	5	6	7	8	5	6	7	8	9	10	7	8	9	10	11	13	+	—
P	8	5.0						1	1				1	2	2	1			5	1
	6	5.0	5.0			5.0						1		1	2	1	1		5	
	6	.5	.5			.5			2					1	1	2			4	
P + Ac	6 P	.5			.5				1		2				1	2			3	
	Ac	.4			.4															
E	8	.5		.5		.5	2			2	1							3	1	2
	8	1.0					1	5	1							1			1	
B	8	.1	.5			.5		4					2		2					4
S	8	.1	.5			.5				2					6					6
Totals	58						3	10	5	4	3	1	3	4	14	7	1	3	19	13

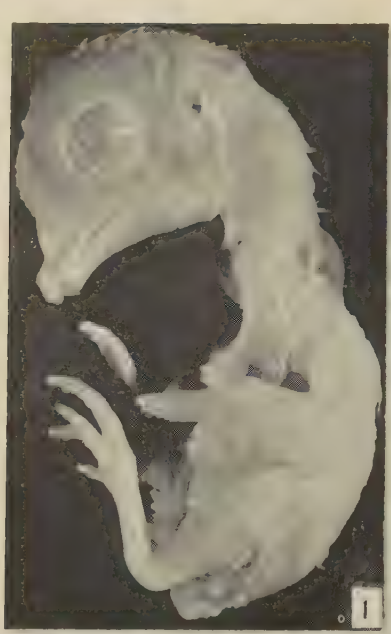
S, 0.85% saline controls; B, 0.10% boric acid; Ac, acetylcholine; Sn, syndrome produced with prostigmine bromide or eserine salicylate (Fig. 4 and 5).

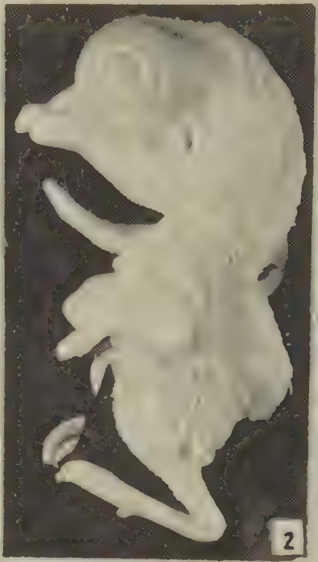
kyphotic animals, the extensive hypoplasia and atrophy of anterior horn cells was most striking. A reduction in volume of musculature was observed along the vertebral column and in the lower extremity. Spinal and sympathetic ganglia were well differentiated but often fused into continuous masses in regions of severe bending. Pedicles and laminae of sacral and coccygeal vertebrae were usually missing. This was generally associated with absence of the spinal cord. Although the brain and brain stem were reduced in size, no gross abnormalities were observed.

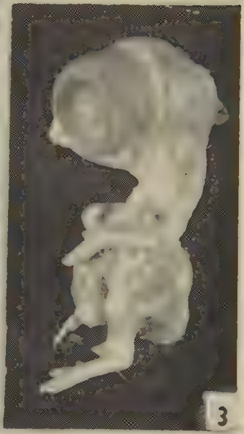
Above experiments were supplemented with observations on 12 embryos injected on the fourth day with .05 mg of physostigmine salicylate. Nine survived 8-12 days' incubation. Of these, 2 were normal, 4 with slight kyphosis at thoracic and lumbar levels; one with normal vertebral column but with shortened lower beak, retarded eyelids and micromelia (Fig. 6); one with no coccyx and with shortened extremities. With high dosages it was typical to find abnormalities in combination with kyphosis. When weaker dosages (.05 mg) were given, the abnormalities were scattered, appearing singly or in unpredictable combinations.

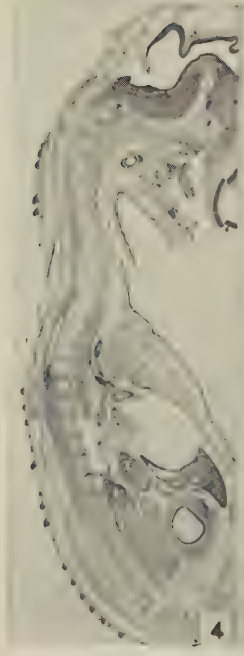
Discussion. Landauer(3) reported the morphological consequences of treatment with sulfanilamide, eserine, and insulin. With heavy doses of these drugs, shortened beaks, micromelia, syndactylism, clubbed down and microphthalmia were observed. Weaker doses produced abnormalities of less degree and a scattering of abnormalities, observed singly or in various combinations. His results were partly confirmed with our observations on embryos treated with weak doses of prostigmine bromide or physostigmine salicylate (Fig. 6). Since similar abnormalities were reported in embryos treated with sulfanilamide and insulin, the mechanism indicated is probably of a non-specific nature.

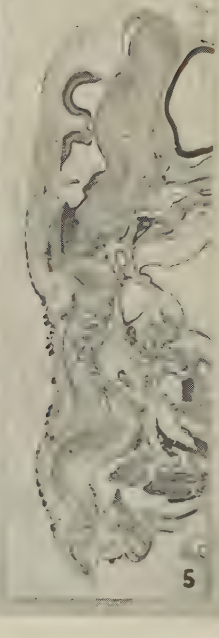
Observations were made by Sawyer(6) on amblystoma embryos reared in various concentrations of eserine (0.01%-0.001%). At Harrison stage 46 a majority of eserinized animals developed one or 2 sharp curvatures of the trunk. There was little retardation of growth, digits were normal, length of gills and height of dorsal fin were decreased. The width of the head was increased. Curvatures of the trunk did not disappear after decapitation or destruction of the spinal cord. This was interpreted as evidence of the muscular origin of the bend reaction. Removal of animals from the inhibitor solution and transfer to water did not eliminate the bend reaction. This persisted as though the skeleton had become fixed in position. Chick embryos eserinized at 4-8 days of incubation subsequently developed extensive kyphosis observed at 7 to 13 days' total incubation. Although this condition seemed more severe than that described for amblystoma by Sawyer(6), the mechan-











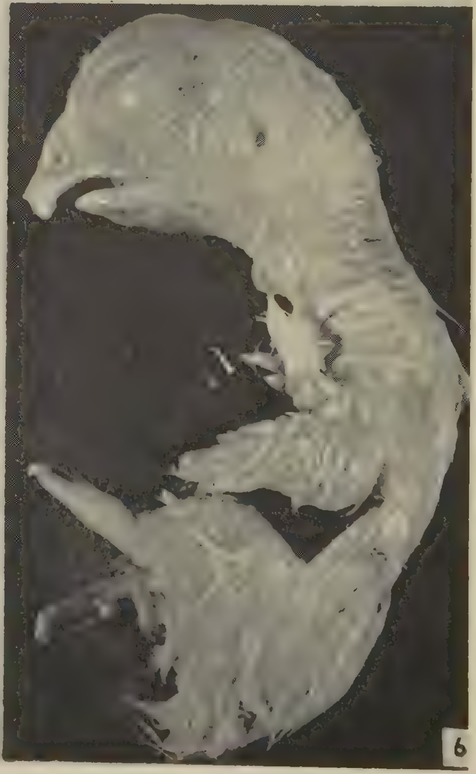


FIG. 1. Control embryo (13-d) treated (yolk sac) on 4th, 5th and 8th days of incubation with 0.1, 0.5 and 0.5 cc of 0.85% saline. ×2.

FIG. 2. Embryo (P6, 13-d) treated (yolk sac) on 4th, 5th and 8th days of incubation with 5 mg of prostigmine bromide; total dosage 15 mg. ×2.

FIG. 3. Embryo (P1, 8-d) treated (yolk sac) on 4th day of incubation with 5 mg of prostigmine bromide. ×2.

FIG. 4. Mid-sagittal section of control embryo (12-d) treated (yolk sac) on 4th, 5th and 8th days of incubation with 0.1, 0.5 and 0.5 cc of 0.85% saline. ×3.4.

FIG. 5. Mid-sagittal section of embryo (12-d) with extensive kyphosis showing 3 compensating S-shaped curvatures. Treated (yolk sac) on 4th, 5th and 8th days of incubation with 5 mg of prostigmine bromide; total dosage 15 mg. ×3.4.

FIG. 6. Embryo with micromelia and shortened lower beak (E-10, 13½-d). Treated (yolk sac) on 4th day of incubation with 0.05 mg of physostigmine salicylate. ×2.

ism of formation may be similar.

It is interesting that the treatment of embryos with large doses of acetylcholine during 4 to 12 days of incubation did not result in malformations similar to those obtained with cholinesterase inhibition. This would suggest the destruction of acetylcholine shortly after its administration. Since cholinesterase increases rapidly as the neuromuscular system develops this is entirely possible. Atropine, a cholinergic block, had no deleterious effect on development.

Summary. 1. Large doses of acetylcholine (1.20-2.85 mg) were injected in the yolk sac, air chamber, or directly on the embryo during the 4-12-day incubation period. These doses were well tolerated by the embryo and attended with no gross abnormalities. Atropine similarly administered produced no malformation. 2. The yolk sac of 4-day embryos was injected with weak doses (0.05 mg) of physostigmine salicylate. Shortened beaks, micromelia, and retarded development of eyelids were observed at 8 to 13 days' incubation. Inasmuch as similar anomalies were reported in sulfanilamide and insulin treated animals, these anomalies cannot be considered eserine specific. 3. With larger doses (1-15 mg) of prostigmine bromide or physostigmine salicylate, the above mentioned anomalies were generally associated with severe midline bendings of the vertebral column (Figs. 4,5), and this was interpreted as specific for cholinesterase inhibition.

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Extraction and Concentration of Thromboplastic Material from Human Urine. (22321)

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Although human urine is known to have thromboplastic property(1,2), little attention has been given to the concentration or purification of the urinary component responsible for this property. There is described herein a simple method for extraction and concentration from normal urine of material with thromboplastic activity, and some characteristics of this material are discussed.

Material and methods. Pooled urine obtained without preservative from normal men is used. The urine, refrigerated not longer than 2 days, is filtered through nonabsorbent cotton, and the pH brought to 4.5 with 2 N HCl. Five g of $BaSO_4$ (x-ray quality) is added to each liter of urine, and the mixture is stirred for 15 min. without being allowed to foam. It is then filtered through large Berkefeld candles type N and the filtrate discarded. Thromboplastic material and a fibrinolysokinase are absorbed on the $BaSO_4$. The $BaSO_4$ is mixed with distilled water (one volume for every 20 volumes of urine), and stirred for 15 min. at a pH of 5.2. The mixture is next centrifuged (20 min. at 1060 *g*). This elution is repeated. The eluates are combined and the sediment set aside for elution of fibrinolysokinase with 3.8% Na-citrate, if desired. The clear, almost colorless eluates are then dialyzed in the cold for two days against distilled water; a slight turbidity forms. All further procedures are carried out at 5°C. The pH is adjusted by a glass electrode to 3.5, and a precipitate is formed. This is collected by centrifugation after standing for one hour and is then dissolved in 1/1,000 of original volume of urine with 1/100 M glycine buffer of pH 9, stirred for 10 min., and stored for one hour. Then the preparation is centrifuged for 30 min. at 2000 *g*. The supernatant is dialyzed by a continuous rocking device, in a cellophane (8/32 inch, Nojax Casing Visking) tubing against running CO_2-free distilled water until pH of 7 is reached. When the supernatant is lyophilized, a white powder soluble to the extent of 1% in 1/100 M glycine buffer of pH 9 is obtained. In solution, it forms a colorless, viscous, slightly opalescent fluid which can be heated to the boiling point (96.5° in Denver) (Table I) with relatively slight loss of thromboplastic activity. This substance is subsequently referred to as the "T" (thromboplastic) fraction. Yield is 2-3 mg/liter.

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* This work was done in part during tenure of Research Fellowship of Amer. Heart Assn. Supported by grants-in-aid from American Heart Assn. and the Belle Bonfils Memorial Blood Bank, Denver.

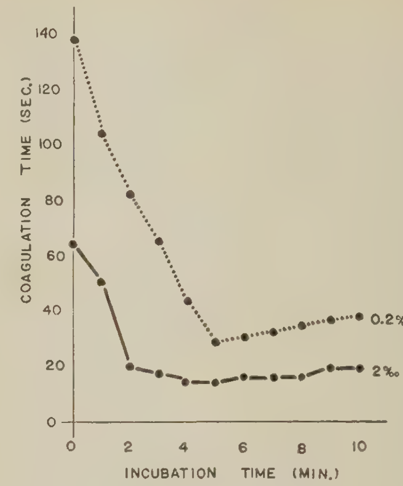

FIG. 1. Thromboplastin generation test. Platelet suspension is replaced by "T" fraction in 1/100 M glycine buffer, pH 9.

Properties. Action of the "T" fraction on hemophilic plasma under various conditions is summarized in Table I. A concentration of only a few micrograms of the "T" fraction per cc of hemophilic plasma is required to restore the clotting process to normal. Heating affects activity of the "T" fraction only to a minor degree, whereas prolonged high-speed centrifugation removes the active material.

The "T" fraction is similar in behavior to

TABLE I. Effect of "T" Fraction on Clotting of Hemophilic Plasma under Various Experimental Conditions. 6.6 μg "T" fraction added/cc plasma.*

Treatment	Recalcification time after addition of "T" fraction (min.)	Prothrombin serum time after addition of "T" fraction (sec.)
2% in glycine buffer, pH 9	9	34.5
Idem; centrifuged 90 min. at 31,000 *g*	46	20
2% in glycine buffer, pH 9	9.3	54
Idem; heated in sealed ampules at 96.5°C, 20 min.	9.5	45

* One μg corresponds to 0.3 ml urine.

Plasma from 2 different subjects has been used to obtain the data for this table. Control recalcification time and prothrombin serum time were 157 min. and 19 sec., respectively, for the first 2 experiments and 58 min. and 20.2 sec. for the last two.

brain extract since both substances contain an incompletely activated thromboplastin. The thromboplastin-like activity of the "T" fraction can be considerably enhanced by incubating it with serum and prothrombin-free plasma, as is done in the thromboplastin generation test(3). Fig. 1 shows that the "T" fraction becomes fully activated after incubation for 4 min. with serum, together with plasma treated with $BaSO_4$.

Discussion. One difference between the "T" fraction and brain thromboplastin is that the latter, when heated, has no effect on the prothrombin consumption of hemophilic blood (4). Another difference is that the "T" fraction can be centrifuged at 2000 *g* for 30 min. without an appreciable reduction in activity. A centrifugal force of 31,000 *g* for 90 min.—conditions similar to those used by Chargaff (5) to purify tissue thromboplastin—is required in order to precipitate this material. Biggs, Douglas and Macfarlane(6) have previously shown that brain extract can be activated by incubation with serum and prothrombin-free plasma—a phenomenon also demonstrable with the "T" fraction. Further studies are indicated in order to determine whether tissue thromboplastin is identical with urine thromboplastin.

Neither the physiologic significance nor the source of the urinary thromboplastic material is known. Using the present method for purification, it is possible to obtain thromboplastin from the very same individual on whom studies are to be performed. Thus, it is no longer necessary to resort to heterologous materials in studies of clotting.

Summary. A method is described whereby thromboplastic material is extracted from normal male human urine by adsorption on $BaSO_4$ and subsequent elution. The thromboplastic material is comparatively resistant to heat; its activity is enhanced by incubation with serum and prothrombin-free plasma. As little as 6 μg/cc of the thromboplastic material per cc of hemophilic plasma was found to restore the clotting properties of this plasma to normal.

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Metabolic Interrelationship between Ascorbic Acid and Pteroylglutamic Acid. (22322)

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A close interrelationship between metabolism of ascorbic acid and pteroylglutamic acid (PGA) was reported by various workers (1-4). Schwartz and Williams(5-7) observed that in PGA deficiency produced by feeding aminopterin, rats excreted less ascorbic acid in urine, and their liver ascorbic acid contents were diminished. This indicated that biosynthesis of ascorbic acid was dependent on PGA-nutrition of animals. Chloretone enhances the biosynthesis of ascorbic acid in rats(8). It was, therefore, of interest to find out if the increased synthesis of ascorbic acid, after chloretone treatment, was interfered with in rats treated with aminopterin. Ascorbic acid helps in the conversion of PGA to citrovorum factor (CF) in rats(9). It was also of interest to find out if this conversion was further stimulated in chloretonised rats.

Methods. Biosynthesis of ascorbic acid. Male rats weighing 100-170 g were fed mineralized whole milk with 2 drops of concen-

TABLE I. 24-Hour Urinary Excretion of Ascorbic Acid by Rats in mg. (Avg excretion of 12 rats.)

	1 day	3-4 day	6-7 day	8-10 day
Milk diet alone	2.5	2.4	2.5	—
+ a*	1.4	1.5	1.4	.7
+ c	1.9	10.4	11.1	11.0
+ c + a	1.9	10.6	12.0	10.4
+ s + c	1.9	9.7	13.2	12.0

* a = aminopterin; c = chloretone; s = sulfaguanidine.

trate of vitamin A and D (Adexolin-Glaxo), twice a week. The animals were divided into 5 groups of 12 animals each. Group I received milk without supplement. Group II were fed 40 γ aminopterin per day. Group III were fed daily 20 mg chloretone dissolved in .2 cc groundnut oil. Group IV were fed both aminopterin and chloretone in doses mentioned above, and Group V were fed daily chloretone and .5 g sulfaguanidine. Within 7 days rats fed aminopterin showed deficiency syndromes. Each animal was placed in a metabolism cage over a glass funnel and urine collected in conical flasks containing 2 cc of a 10% solution of oxalic acid and 1 cc toluene. Total ascorbic acid in 24 hour output of urine was determined by titration against a standardized solution of 2,6-dichlorophenol indophenol(10). Animals of the different groups were killed on tenth day of experiment and ascorbic acid present in liver, kidneys, adrenals, small intestine, testis and pancreas was extracted with 4% sulfosalicylic acid and determined by titration with 2,6-dichlorophenol indophenol(11). Dehydroascorbic acid was absent from these tissues. Thiamine, riboflavin and nicotinic acid contents of liver of all animals were determined(12-14). No significant change in these values in livers from different groups of animals could be observed. The results are given in Tables I and II. *Excretion of citrovorum factor and ascorbic acid.* Ten male rats were divided into 5 pairs, each placed in one metabolism cage and urines collected as described above except when analysed for CF. For CF estimation urines were collected under toluene only. Rats were fed mineralized milk supplemented with 100 γ PGA by stomach tube per animal daily for 2 weeks. Ascorbic acid and CF were estimated in urine samples on alternate days. CF was estimated by the method of Sauberlich and Baumann(15) using leucovorin (Lederle) as the reference standard. Results are given in Table III. Male rats of another group of 12 were similarly fed 100 γ PGA for one week and urinary excretion of CF and ascorbic acid determined. After one week these animals were fed 20 mg chloretone in addition to PGA and urinary excretion of CF and ascorbic acid were determined. The results are given in Table III. *Estimation of CF, ascorbic acid and glutathione contents of livers.* At the end of 2 weeks, rats from each group, described in previous section, were killed by decapitation, livers removed, chilled, weighed, ascorbic acid and glutathione estimated according to the method described previously (11) in aliquots of liver samples and CF according to the method of Doctor *et al.*(16) after incubating aliquots of liver homogenate in nitrogen. The results are given in Table IV.

Results. Administration of aminopterin diminished urinary excretion of ascorbic acid indicating interference in normal synthesis of ascorbic acid in PGA deficiency. Urinary excretion of ascorbic acid was stimulated by administration of chloretone either alone or in conjunction with aminopterin. PGA deficiency, therefore, did not hamper urinary excretion of ascorbic acid in chloretonized

TABLE II. Ascorbic Acid Values of Tissues of Rats (mg/100 g Fresh Tissue).

Rats on	Milk	Milk + a	Milk + c	Milk + c + a	Milk + c + s
Liver	31.2 ± .9*	23.8 ± .4	43.1 ± 2.3	35.8 ± 3.2	42.1 ± 2.2
Kidney	20.9 ± 1.1	15.3 ± 1.4	34.8 ± 1.6	23.5 ± 3.2	35.0 ± 1.7
Adrenals	413.7 ±17.5	274.0 ±14.2	408 ±34.4	294 ±44.5	392 ±46.4
Intestine	46.2 ± 1.2	35.6 ± 2.0	59.8 ± 2.8	45.9 ± 5.1	60.9 ± .6
Testis	24.4 ± .7	23.6 ± .7	26.4 ± 1.0	28.6 ± .1	27.5 ± .4
Pancreas	9.6 ± .4	8.0 ± .7	12.9 ± 1.3	10.4 ± .9	10.8 ± 0

* Stand. error.

TABLE III. 24-Hour Urinary Excretion of CF and Ascorbic Acid by Rats Fed 100 γ PGA per Day.

	Before chloretone		After chloretone	
	CF excretion/animal/day	Ascorbic acid excretion/animal/day	CF excretion/animal/day	Ascorbic acid excretion/animal/day
1st wk	468 ± 44 mγ	.62 ± .06 mg	—	—
2nd wk	566 ± 26	.65 ± .06	1572 ± 163 mγ	6.75 ± .63 mg

TABLE IV. CF, Ascorbic Acid and Glutathione Values of Rat Liver.

	Without chloretone	With chloretone	t
Ascorbic acid	24.3 ± 1.2 mg/100 g	34.6 ± 1.4 mg/100 g	4.7
Glutathione	233.7 ± 8.8 ”	273 ± 13.9 ”	7.5
CF incubated in nitrogen	151 γ/100 g	143 γ/100 g	

rats. Sulfaguanidine fed rats receiving chloretone also excreted in the urine increased amounts of ascorbic acid. The intestinal flora, therefore, may not be concerned in increased urinary excretion of ascorbic acid.

Ascorbic acid contents of liver, kidney, adrenals and small intestine significantly diminished in animals which received aminopterin. Ascorbic acid contents of these tissues increased when animals were fed chloretone. Administration of aminopterin in addition to chloretone increased ascorbic acid values of these tissues, but the values were at a lower level as compared to values in animals which received only chloretone. These findings indicated that aminopterin-induced PGA deficiency interfered with biosynthesis of ascorbic acid by normal and chloretone-fed rats. There was, however, no change in ascorbic acid contents of testes and pancreas in rats differently treated.

After administration of PGA in normal rats there was 6- to 8-fold increase in urinary excretion of CF. This excretion was further enhanced when animals were fed chloretone. Ascorbic acid and glutathione contents of liver were increased in chloretonized rats but CF contents did not change appreciably. The increased conversion of PGA to CF in chloretone-fed rats might be due to increased synthesis of ascorbic acid and glutathione. Ascorbic acid enhanced urinary excretion of CF in rats fed PGA(9).

Summary. The effect of aminopterin on ascorbic acid excretion and tissue contents of ascorbic acid was studied in both normal and chloretone-fed rats. Aminopterin decreased urinary excretion of ascorbic acid but did not decrease excretion in chloretone-fed rats. Sulfaguanidine also could not diminish the increased ascorbic acid excretion in chloretonized rats. Ascorbic acid contents of liver, kidney, adrenals and small intestine significantly diminished in both groups of animals receiving aminopterin alone or in conjunction with chloretone. Ascorbic acid contents of testes and pancreas of differently treated rats did not change. Chloretone feeding enhanced urinary excretion of ascorbic acid and CF and increased ascorbic acid and glutathione contents of liver.

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In vitro Cultivation of Porcine Kidney. (22323)

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In early tissue culture experiments, choice of a menstruum for proliferation of a virus was generally restricted to host and tissue normally parasitized by the given virus. It is now apparent that these restrictions are not so clearly defined. Enders and his co-workers (1) for instance, propagated poliomyelitis virus in non-neural tissues (kidney, skin-muscle and intestine) and Maitland and Laing (2) grew vaccinia virus on rabbit kidney.

Recent observations that epithelial cells of renal origin support multiplication of a number of viruses beyond the confines of host or tissue specificity prompted us to study the nutritive factors involved in culturing of kidney tissue. Kidney of porcine origin was used because of favorable economic considerations and its availability.

Materials. A. *Porcine Constituents.* 1. *Hog Embryo Extract (HEE).* Embryos (10-20 cm long) were removed from the gravid uteri of sacrificed sows and washed with cold physiological saline. The tails, hooves, heads, ribs, and intestinal tracts were removed and the remaining skin-musculature and organs were cut into small pieces and placed in the deep freeze. The dismembered embryos were thawed 24 hours later and homogenized in a Waring Blendor for 30 seconds. An equal volume of Hanks Balanced Salt Solution containing antibiotics was added and the mixture rehomogenized an additional 15 seconds. The homogenized embryo extract was clarified by cheese cloth filtration and by centrifugation (4,000 rpm for 45 minutes at 1°C). The HEE was sterilized by filtration through an EO (Ertel) pad and stored in the deep freeze in 50 ml amounts. 2. *Hog Serum (HS)* was Seitz filtered and stored in deep freeze in 10 ml amounts until used. 3. *Hog Serum Ultrafiltrate (HSU)* was prepared by forcing hog serum through Visking casing. The pH of HSU was adjusted to 7.3 by means of 0.1 M NaH_2PO_4. The HSU was then Seitz filtered and stored in deep freeze in 10 ml amounts. (Selection of kidney tissue and hog constituents should be made from healthy non-immune stock, otherwise, humoral antibody or endogenous viruses may interfere with virus proliferation.) B. *Bovine Constituents.* Beef embryo extract (BEE), serum (BS), and serum ultrafiltrate (BSU) were obtained from Microbiological Associates, Bethesda, Md. C. *Balanced Salt Solution (BSS)* used in preparation of the media was prepared in the manner specified by Hanks and Wallace(3). Just previous to use, 2.5 ml of a 1.4% solution of sterile $NaHCO_3$ was added/100 ml of BSS. The pH of BSS was approximately 7.4. D. *Synthetic Mixture #199 (SM #199)*, composed of inorganic salts, amino acids, vitamins, nucleic acid derivatives and lipids, was prepared in accordance with procedure recommended by Morgan, Morton and Parker(4). SM #199 was refrigerated until used. E. *Nutritive Media* for initiation of cellular growth consisted of isotonic saline solution (either BSS or SM #199) together with varying amounts of serum, serum ultrafiltrate and embryonic extract. The media were compounded and used within 24 hours. F. *Maintenance Media* were used for cellular maintenance: Hanks-Simms medium was prepared by combining one part of sterile serum ultrafiltrate with 3 parts of BSS. Tenth molar NaH_2PO_4 was used to adjust the pH to 7.5. SM #199 was used without further adjustment. G. *Antibiotics.* Crystalline penicillin G (the potassium salt) and streptomycin (calcium chloride complex) were employed routinely in all media at levels of 250 units and μg/ml respectively. H. *Chick Embryo Extract (CEE)* was prepared in accordance with directions of Syverton *et al.*(5). I. *Hog Serum Fractions* II, IV, V and IV + V were prepared according to methods described by Cohn *et al.*(6) or Cammarata and Deutsch (7).

Methods. An entire kidney was removed from young hog (40-60 lbs. weight) using aseptic technic. The kidney was washed with BSS containing antibiotics. Capsule and macroscopic vasculature were removed and a pie shaped portion consisting of cortical and medullary substance was minced in a Petri dish containing several ml of BSS or SM #199. Tissue fragments were reduced to approximately 1 mm and washed several times with BSS or SM #199 until supernatant was clear. A. *Plasma Clot Technic.* The lower two-thirds of each culture tube (16 x 150 mm) was coated with 0.05 ml chicken plasma (Microbiological Associates). Using a platinum wire inoculating loop, 6-8 fragments of tissue were planted in a single vertical row at 10-15 mm intervals in the plasma coated area. Five hundredths ml of CEE was added and distributed over the tissue embedded in the plasma to induce coagulation. Excess CEE was allowed to drain. Following complete coagulation, 1 ml of nutritive medium was added to each tube. The culture tubes were then capped with white "non-toxic" rubber stoppers (The West Co.) and incubated at 36°C in a roller drum revolving at rate of 12 revolutions/hour. B. *Dry Tube Technic.* Minced tissue suspended in small amount of maintenance medium was introduced into lower half of prewarmed (45°C) test tubes by Wright pipettes. The tubes were inverted to effect drainage and stored at 4°C for 30 minutes. The tubes were then reinverted and 1 ml of nutritive medium was added to each tube. Tubes were stoppered and incubated at 36°C in a roller drum. The dry tube technic was used almost exclusively throughout the investigation when preliminary studies revealed that growth exceeded that of the plasma clot technic while granulation was almost nonexistent. C. *Fluid Change.* When growth was well established (usually within 5 to 7 days) the nutritive medium was removed. Each culture was rinsed twice with 1 ml aliquots of BSS or SM #199. One ml of maintenance medium was added and cultures were reincubated for 5 additional days. The maintenance fluid was changed at intervals of from 4 to 7 days depending upon pH of the medium.

Results. The nutritive media recommended for proliferation of cells in tissue culture are numerous, and vary as to their constituents and percentages in accordance with the organ and species cultured(8,9,10,11,12). To determine the influence of homologous and heterologous constituents of nutritive media on propagation of hog kidney epithelial cells, cultures were grown in media consisting of both beef and hog serum, serum, serum ultrafiltrate and embryo extract. The composition of the various media as well as their influence on growth of hog kidney epithelial cells are shown in Table I. The data derived from this initial study indicated that an all hog nutritive medium was superior to an all beef system and the introduction of any hog constituent into a nutritive medium significantly enhanced rate and extent of growth of hog kidney epithelial cells. Furthermore, growth in a medium composed of hog serum (10%) and SM #199 (90%) excelled that of any

TABLE I. Composition of Nutritive Media Used for Proliferation of Hog Kidney Epithelial Cells in Tissue Culture and Their Influence on Rate and Extent of Growth.

Medium constituent	% composition	Medium No. 1	2	3	4	5	6	7
Serum ultrafiltrate	17.6	B*	H*	H	B	B	—	—
Serum	20.0	B	H	B	H	B	—	—
Embryo extract	10.0	B	H	B	B	H	—	—
Balanced salt sol.	52.4	+	+	+	+	+		
Serum	10.0	—	—	—	—	—	B	H
Synthetic mixture #199	90.0	—	—	—	—	—	+	+
		pH 7.4					pH 7.6	
% of cultures growing†		26	82	75	78	74	56	88
Extent of growth‡		1+	2+	2+	2+	2+	2+	4+

* B—Beef origin; H—Hog origin.

† Within 7 days.

‡ Extent of cellular outgrowth based upon No. of layers of cells radiating from the plant and was evaluated in terms of 1 to 4+.

other media tested.

In accordance with these findings, a more definitive experiment was designed to determine optimum combinations and concentrations of hog constituents to be used in conjunction with SM #199 for growth of kidney cells. The results of this study, summarized in Table II, confirm and stress the growth stimulating role of hog serum in the cultivation of epithelial cells. Cultures grown in a medium containing hog serum were consistently more proliferative than cultures lacking that constituent. HEE and HSU, on the other hand, either alone or in combination with HS did little to influence growth. The data also indicate that a minimum of 7.5% hog serum in SM #199 was necessary to insure good growth.

It is interesting to note that none of the media itemized in Tables I or II adequately supported growth of either porcine splenic or testicular fibroblasts.

Having ascertained that porcine serum was a necessary medium constituent, attempts were made to further reduce the complexity of the nutritive medium by substitution of various porcine serum fractions for whole serum. Epithelial cells were consequently cultured in SM #199 containing serum fractions (II, IV, V, or IV + V) in concentrations equivalent to those found in whole serum (8,9). Findings (Table III) indicate that Fraction II did not initiate or support epithelial growth, and that Fractions IV and V, when used independently, produced growth inferior to that of whole serum. However, when Fraction IV + V was used, growth superior to that of whole serum resulted, suggesting a complementary action. The results also indicate the presence of an inhibitor in serum which is inactivated or removed during fractionation procedures.

It is interesting to note that bovine plasma Fractions IV and/or V have been found to enhance growth of monkey kidney cells in tissue culture(13).

Summary and Conclusions. 1. Some of the nutritional requirements for the *in vitro* culti-

TABLE II. Influence of Different Concentrations of Hog Constituents on Growth of Hog Kidney Epithelial Cells in Tissue Culture.

Medium constituent*	Medium No. 1	2	3	4	5	6	7	8	9	10	11	12
Serum ultrafiltrate	10	5	0	0	0	0	0	0	5	5	0	0
Serum	0	0	10	7.5	5	2.5	0	0	5	0	5	0
Embryo extract	0	0	0	0	0	0	10	5	0	5	5	0
Synthetic mixture #199	90	95	90	92.5	95	97.5	90	95	90	90	90	100
% of cultures growing†	50	35	90	100	70	75	0	0	75	15	78	30
Extent of growth	0	0	4+	4+	3+	3+	0	0	3+	0	2+	0

* In %. † Within 7 days.

TABLE III. Influence of Hog Serum Fractions II, IV, V and IV + V on Growth of Hog Kidney Epithelial Cells in Tissue Culture.

Hog serum fractions*	% of cultures exhibiting various degrees of growth 0	1+	2+	3+	4+
II	100	0	0	0	0
IV	11	44	45	0	0
V	43	28	29	0	0
IV + V	0	0	0	0	100
Whole serum	12	13	37	38	0

* In synthetic mixture #199.

vation of hog kidney epithelial cells were determined. The data indicate that cultures grown in a "simple" medium containing Synthetic Mixture #199 (92.5%) and hog serum (7.5%) were consistently more proliferative than cultures cultivated in more complex media containing embryo extract, serum ultrafiltrate and serum of homologous and heterologous origin. 2. None of the media tested adequately supported growth of either splenic or testicular fibroblasts. 3. The influence of various Porcine Serum Fractions (II, IV, V and IV + V) on growth of hog kidney epithelial cells was also investigated. When Fraction IV + V was used, excellent growth was achieved. When Fraction II, IV or V was used independently, growth inferior to that of whole serum resulted.

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Some Metabolic Effects of $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,11,20-trione (Meticorten) Administered Orally to Dogs. (22324)

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The discovery of the glucocorticoid and anti-inflammatory activities of Meticorten ($\Delta^{1,4}$-pregnadiene-17α,21-diol-3,11,20-trione) in experimental animals(1,2) and of its antirheumatic activity in human subjects(3) prompted studies on the metabolic actions of this steroid. Most experimental animal studies with Meticorten have utilized mice or rats. There are, to our knowledge, no published studies of the effects in dogs of large doses of Meticorten on water and nitrogen balance, or of the effects on blood sugar level or on the glucose tolerance curve before and during prolonged oral treatment. The production of a marked polyuria and polydipsia by daily *subcutaneous* treatment of dogs with 10 mg/kg of cortisone has been reported(4). Dogs given the same dose orally did not exhibit these effects. Also, it has been noted(5) that daily intramuscular injections of cortisone (50-300 mg/day) to dogs produced polydipsia and polyuria but there were no changes in fasting blood sugar levels or on the glucose tolerance curves. More recent studies with dogs(6) have confirmed the observations on the absence of effect of cortisone on blood sugar level and also showed a small but apparently significant elevation of R.Q., a decrease in basal metabolic rate, and an increased diuresis and nitrogen excretion during treatment.

Methods and materials. Four intact male beagles were used in this study. Each animal was conditioned to a separate metabolism cage for the entire period of the experiment and was housed under the same conditions for at least 6 months prior to the study. The feeding regimen, diet composition (high nitrogen), and the methods for evaluating nitrogen-balance status have been described previously(7). Meticorten was administered orally, once a day, in a gelatin capsule. Water intake, urine output and specific gravity were measured each morning, and tests for glycosuria and albuminuria were run on aliquots of each 24-hour urine collection. Fasting blood sugar samples were analyzed by a standard method. Oral glucose tolerance tests were run by administering 3.0 g glucose /kg body weight via stomach tube and analyzing blood samples for glucose at 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 hours. The dosage regimen in this study was one in which the steroid dose was increased at intervals until consistent polyuria and polydipsia were obtained and then a gradual reduction of dose was instituted until these changes had disappeared.

Results. Table I summarizes certain of the data obtained with the 4 dogs but does not indicate the rate at which the various changes occur. Fig. 1 and 2 provide some indication of the responses over a period of time and at increasing or decreasing dosage. These figures, representing data for dog No. 18, are quite similar to results obtained with the 3 other dogs used in this study.

It is evident from Table I that with a large dose of steroid it was possible to produce a marked polydipsia and polyuria in all 4 dogs, an increased urinary nitrogen excretion, a decrease in urine specific gravity, and a complete or almost complete eosinopenia. It is also apparent that these effects continued to manifest themselves even as the dose was reduced from the highest level (250 mg/day) to doses of 50-75 mg (Fig. 1).

TABLE I. Effect of Oral Meticorten Treatment on Blood Sugar, Water and Nitrogen Balance.

	Dog No.			
	17	18	31	32
Before treatment (control)*				
Wt, kg	11.6	10.3	7.6	8.0
N, g N/kg/day	E¶	−.07	−.04	E
Water intake, cc†	200	540	520	800
Urine, cc†	200	260	325	420
Specific gravity	1035	1040	1032	1030
Blood sugar, mg %	55	59	62	67
During treatment‡				
Wt, kg	10.8	9.5	6.5	7.2
N, g N/kg/day	−.35	−.27	−.38	−.45
Water intake, cc	1400	1500	1400	1500
Urine, cc	1400	1610	1250	1620
Specific gravity	1022	1010	1012	1008
Blood sugar, mg %	61.2	59	65	53
Max Meticorten dose, mg/day	250	250	250	250
% fall in eosinophils§	100	100	100	100
After treatment‖				
Wt, kg	11.2	9.5	7.0	7.4
N, g N/kg/day	+.04	+.02	+.08	E
Water intake, cc	100	500	410	250
Urine, cc	100	380	315	150
Specific gravity	1035	1035	1040	1025
Blood sugar, mg %	59	71	55	59

* Twelve-day control period.

† Maximum 24-hr value.

‡ Avg values at maximum dose. Period at this dose 12 days for dogs 18, 31 and 32, and 21 days for dog 17.

§ Compared to "Before treatment (control)."

‖ Post-treatment period 17 days for dogs 18 and 31, and 3 days for dogs 17 and 32.

¶ E = equil.

At no time during these experiments did any animal exhibit hypo- or hyperglycemia, glycosuria or albuminuria. Glucose tolerance curves obtained before and during treatment at the maximum dose level (250 mg/day) were normal in every instance.

We have also confirmed (unpublished observations) the published observations on the effects of cortisone on water metabolism. In our experience, the minimal doses which produce significant eosinopenia do not necessarily produce negative nitrogen balance or polydipsia and polyuria. Similarly, larger doses which produce eosinopenia and negative nitrogen balance do not cause polydipsia or polyuria when the dose is not high (100-150 mg/day). However, with still larger cortisone doses (200-300 mg/day) all these effects usually can be produced, although we

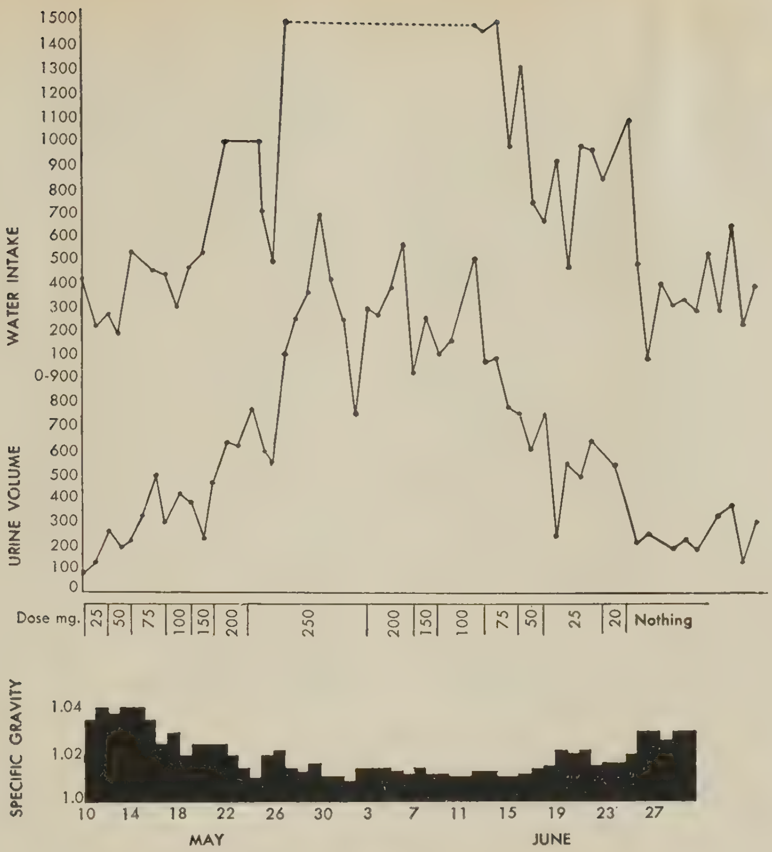

FIG. 1. Effect of Meticorten on water balance and urinary specific gravity in normal dog 18.

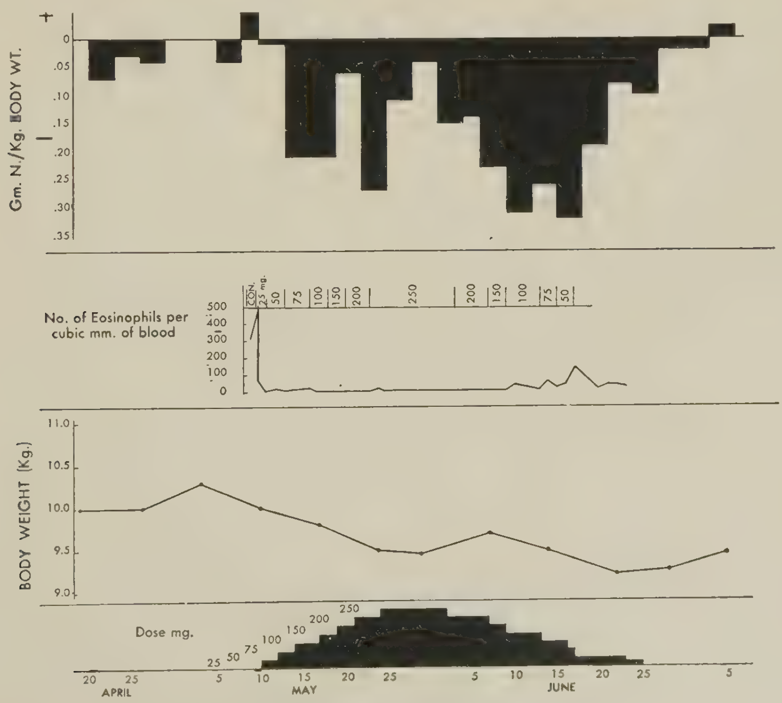

FIG. 2. Effect of Meticorten on nitrogen balance, body wt and eosinophil level in normal dog 18.

have had experience with one male dog which, even at doses producing polydipsia and polyuria as well as negative nitrogen balance, exhibited no change in circulating eosinophil level. Also the sequence in which the various changes (eosinopenia, negative nitrogen balance, polydipsia and polyuria) usually occur would suggest a differential sensitivity of each of these responses to dose.

Summary. 1. In dogs large, prolonged, oral doses of Meticorten induced polydipsia, polyuria, and negative nitrogen balance. 2. Blood glucose levels and glucose tolerance curves were unaffected by the treatment, and no evidence of albuminuria and glycosuria was obtained.

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Protective Action of Glutamine, Cysteine and Other Substances Against Experimental Lathyrism in the Rat. (22325)

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Although the toxic action of sweet peas, *Lathyrus odoratus*, is known to be due to β-aminopropionitrile (occurring as its γ-glutamyl derivative in the sweet pea)(1,2,3), the specific metabolic disturbance caused in the rat by the ingestion of this substance is still undetermined. It is to be expected that discovery of a specific compound having a protective action against sweet pea toxin will aid in revealing the metabolic site at which the toxin acts. The finding of a protective agent may also be of considerable medical importance because of the possible relationship between odoratism (sweet pea lathyrism) and a number of human diseases of unknown etiology(4,5).

Methods. Male, albino, weanling rats, 24-28 days of age and weighing 48-64 g were used in all experiments. All rats were Sprague-Dawley (Holtzman) strain. Because all young rats receiving substantial amounts of sweet pea meal in their diets rapidly develop lathyrism without exception, screening tests for protective agents can be conducted with a relatively small number of rats. For screening purposes, therefore, supplements were usually fed to 3, but sometimes 2, rats at each level. *Diets.* In a few of the earlier experiments the basal diet contained 50% sweet pea meal* and 25% corn starch and was made up as previously described(6). In most of the screening tests, however, the basal diet contained 30% sweet pea meal and 45% corn starch. Supplements were incorporated by replacing an equivalent amount of starch. All diets contained 10% casein (not vitamin-free) except as noted below. In a few instances supplements were added to Rockland Rat Diet containing 0.1% of synthetic β-aminopropionitrile hydrochloride.† Since high levels of casein exhibit some protective action(8,9,10) the following device was used with supplements which appeared to have anti-lathyrismic properties. Five per cent of supplement was incorporated into a diet containing 30%

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* Sweet peas were generously supplied by Mr. Raymond H. Coulter of Ferry-Morse Seed Co.

† β-aminopropionitrile was synthesized by the method of Buc(7), then converted to hydrochloride and crystallized from 95% alcohol.

sweet peas and 30% casein. Rats receiving this diet were compared to rats receiving unsupplemented diet containing 30% sweet peas and 35% casein. If the supplement had appreciable protective activity, it could be expected to cause improvement above that produced by the increment of casein which it replaced.

Evaluation of Supplements. The animals were weighed weekly or oftener. Observations of muscle tone, activity, etc. were made at frequent intervals. The primary criteria for determining the severity of the advancing lathyrism, however, were evaluations of skeletal changes. These were made by palpation of mandibles, spine, and thorax(8). Levels of supplements which cause decreased growth rate sometimes appear to have protective action due to the fact that, other things being equal, the more slowly growing animal develops skeletal deformities at a slower rate. For this reason it was necessary to differentiate between a more slowly developing lathyrism due to decreased food consumption and a positive protective action. Because assessment of severity of the symptoms was subjective, and because there is some individual variation in the rate of progress of the disease, only those supplements which caused an unquestioned improvement definitely outside the symptom complex bracketed by control animals were considered to exert any protective action. Supplements which appeared to be beneficial, but caused an improvement which could conceivably lie within extremes of individual variation, were fed to more rats at the same level as before and at higher levels. Supplements which were found toxic at the levels fed (severe growth inhibition or death) were re-tested at lower levels.

Results. Partial protection shown by high levels of *protein* (casein and gelatin) have already been reported(8). Casein hydrolysate (enzymatic) fed at a 30% level (with 10% casein) showed some protective activity, although it proved to be less protective than casein itself. Lactalbumin was almost equal to casein in protective activity. Zein exerted no protective action. Zein at 30% (with 10% casein), in spite of its amino acid deficiencies, had no growth-inhibiting effect; its effects on growth and on development of lathyrism could not be distinguished from that of starch.

When 5% *L-cysteine-HCl* was incorporated into the basal diet, some inhibition of growth resulted. A 4% level permitted growth comparable to that obtained with the unsupplemented basal diet. Delayed development especially of mandibular exostoses could readily be distinguished by palpation for the first 2 or 3 weeks after weanling rats were placed on diet. This protective action was temporary, however, for at 3 to 4 weeks cysteine-fed rats were no better off than controls, *i.e.*, cysteine did not decrease eventual severity of the skeletal changes but only delayed their onset. At 2.5% cysteine-HCl gave only very slight, barely perceptible, protection. When L-monosodium glutamate (10%), L-monosodium glutamate plus glycine (10% + 5%), L-leucine (10%), L-lysine-HCl (10%), glucosamine-HCl (5%), or pyridoxine-HCl (0.5%) were added to the basal diet containing 4 or 5% of L-cysteine-HCl, no protection over that due to cysteine was obtained. A diet containing 5% cysteine-HCl plus 30% casein gave somewhat better protection than a diet containing 35% casein without added cysteine. The high level of casein in this diet overcame the growth-depressant effect of cysteine. A level of 5% of cysteine-HCl gave significant protection also to rats receiving 0.1% of β-aminopropionitrile-HCl in Rockland Rat Diet, although the growth-depressant effect of the cysteine was here again in evidence. Addition of 5% *L-glutamine* to basal sweet pea diet also caused delay in onset of skeletal changes. In contrast to the growth-depressant effect of cysteine, glutamine had a growth-stimulating effect. The protective action of glutamine was of longer duration than that of cysteine. Five weeks after beginning of experiment, although skeletal deformities by this time were marked, rats receiving glutamine were not only larger than control rats, but their fur was sleeker, they had better muscle tone and were more active. A 10% level of glutamine gave no more protection than did a 5% level. A diet containing 5% glutamine plus 30% casein seemed to give slightly better protection than a diet containing 35% casein without added glutamine, however the beneficial effect of glutamine in this diet was not clear-cut. Glutamine (5%) added to Rockland Rat Diet containing 0.1% β-aminopropionitrile delayed skeletal changes during the first 2 weeks of the feeding trial. After 3 weeks on this diet, however, there was no detectable difference between these rats and rats receiving the same diet without added glutamine. Rats receiving Rockland diet containing the aminopropionitrile grew better than rats receiving sweet pea diet. Glutamine had no growth-stimulating effect in the Rockland diet.

Addition of 5% L-cysteine-HCl plus 5% L-glutamine to basal sweet pea diet had growth-stimulating effect, but protection against skeletal changes was no greater than with either of these supplements alone. Pyridoxine-HCl seemed to vitiate the beneficial effects of glutamine; rats receiving 5% L-glutamine plus 0.5% pyridoxine did no better than rats receiving the unsupplemented basal ration.

The following *amino acids* were not protective: L-lysine-HCl (10%), L-leucine (10%), L-cystine (5%), L-monosodium glutamate (up to 20%), L-glutamic acid (10%), L-ammonium glutamate (10%), L-histidine-HCl (5%-growth inhibition), DL-aspartic acid (10%—some growth inhibition), L-asparagine (10%), β-alanine (5%), DL-methionine (2%; 10%—toxic), glycine (5%), and DL-Serine (4%; 10%—toxic). The partially protective action of lysine reported by others (11) was not confirmed in this study.

The following *carbohydrate derivatives* were found to have no protective action: glucosamine hydrochloride (5%; 15%—toxic), glucuronic acid lactone (5%), chondroitin sulfate (5%), glucosamine-HCl plus chondroitin sulfate (5% + 5%), and sodium glucuronate (10%). Since there was some question as to whether or not glucosamine-HCl exerted slight protective action at the 5% level, and because cysteine was later found to be somewhat protective, glucosamine-HCl and L-cystine HCl (5% + 5%) were fed together. Glucosamine did not provide any protection above that due to cysteine.

None of the following *vitamins* gave any protection: pyridoxine-HCl (0.5%), Ca pantothenate (various levels up to 5%), L-ascorbic acid (50 mg/100 g), Ca pantothenate plus L-ascorbic acid (20 mg + 50 mg/100 g). Liver powder (1:20, GBI) at 4% level plus added pantothenate and ascorbic acid was ineffective.

The following *miscellaneous supplements* gave no protection: monoethanolamine-HCl (up to 10%—levels of 5% and above caused severe growth inhibition), taurine (5%), sodium acetate (5%), sodium propionate (5%), sodium pyruvate (1.5%), betaine (1%), brewers yeast (15%; 30%—toxic), ribose nucleic acid (10%), sodium glycollate (2%), sodium thioglycollate (up to 1%—growth inhibition), sodium thiocyanate (up to 0.5%), ammonium chloride (4%), prostigmine bromide[‡] (up to 3.5 mg/100 g), glutathione (100 mg of monosodium salt/day by subcutaneous injection), and cortisone acetate (0.5 mg/day by subcutaneous injection). An alcoholic extract of casein was prepared by continuously extracting casein with 95% ethyl alcohol for 8 hours. When this extract was added in amounts equivalent to 100 g of casein per 100 g of basal diet (modified to contain 30% casein) no additional protection was obtained.

Discussion. The metabolic site at which sweet pea toxin acts is still obscure. That the metabolism of sulfur is in some way involved seems likely. Protein synthesis itself does not seem to be disturbed, since the animals grow relatively well until the terminal phase of the disease is approached and since no toxic lesions can be demonstrated in liver, kidney or other organs(4,13).

Glutamine also appears to be involved in the metabolic defect. Because glutamate does not seem to be able to substitute for glutamine, it might be inferred that the presence of the amide group of glutamine is critical. However, the interference is not merely one of γ-amidation of glutamate, because methionine sulfoxide, which is known to interfere with γ-amidation of glutamic acid, does not give rise to a syndrome comparable to odoratism even when administered to rats in large doses (rats fed 1% methionine sulfoxide grew normally).§ A clue to the problem may lie in recent publications of Boström, Rodén, and Vestermark(14,15) who reported that glutamine, but not glutamic acid, appears to act as an accelerator of chondroitin sulfate synthesis and probably plays a part in biosynthesis of mammalian mucopolysaccharides. In this connection, it has been pointed out repeatedly that sweet pea lathyrism in the rat seems specifically to involve mesodermal tissues and that the metabolic defect appears to be locallized in the ground substance, which is rich in mucopolysaccharides (*cf.*, for example, 4 and 16).

[‡] Prostigmine bromide was generously supplied by Dr. M. J. Schiffrin, Hoffmann-LaRoche, Inc. Prostigmine methylsulfate has been reported to be effective in treatment of human lathyrism(12).

Conclusions. Of a wide variety of supplements tested for their protective action against development of skeletal deformities in rats fed sweet peas or β-aminopropionitrile, none gave complete protection. Only L-cysteine-HCl, L-glutamine, and proteins were partially protective. Of the proteins tried, casein, gelatin, lactalbumin and casein hydrolysate gave some protection; zein did not. The protective action of glutamine may be related to its probable role in chondroitin sulfate and mucopolysaccharide synthesis.

Note added in press. Experiments completed since this paper was submitted for publication indicate that the protection afforded by L-cysteine-HCl is prolonged if the diet is made up fresh weekly. Under these conditions the protection against development of skeletal changes was greater with cysteine than with glutamine. The decreased protective activity of L-cysteine in an aging diet is probably due to a gradual loss of cysteine because of the lability of its sulfhydryl group.

The author is grateful to Mr. Ata Wuhush who helped with the preparation of diets and with care and feeding of the animals.

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§ Unpublished data.

Relation of Brain Stem to Renal Electrolyte Excretion.* (22326)

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Experimental evidence that a cerebral lesion might affect formation of urine was first presented by Claude Bernard. He produced polyuria without glycosuria in the experimental animal by puncturing the median eminence of the floor of the fourth ventricle

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slightly cephalad to the point where puncture caused polyuria and glycosuria(1). The author's interest in the relationship between the lower brain stem and renal excretion of electrolytes was aroused by study of a patient with probable "cerebral salt-wasting syndrome," due to tumor within the fourth ventricle of the brain. The syndrome consists of persistent loss of sodium and chloride in the urine, despite low concentration of these ions in the blood. In 4 of 6 cases in medical literature (excluding tuberculosis and tuberculous meningitis), patients had diffuse diseases of the central nervous system(2,3). One patient had glioma of the posterior thalamus, with an area of demyelination extending down the brain stem(4). In the sixth case, a brain tumor was present, involving the floor of the fourth ventricle just rostral to the obex(2).

These cases raise the question whether the lower brain stem may exert any control over electrolyte excretion. The following experiment was carried out in an attempt to clarify this problem.

Method. Ten adult female mongrel dogs were used. Anesthesia was induced and maintained by intravenous pentobarbital. An indwelling urethral catheter was left in place throughout each experiment. When intravenous fluids were administered, a continuous gravity drip was started immediately after catheterization. Using aseptic technic, the *cerebellar fossa was opened.* The lower portion of the cerebellar vermis and the medulla were exposed. The lower portion of the fourth ventricle was visualized by retraction or resection of the lower vermis (results were the same whether or not the vermis was resected). Stimulation of the caudal portion of the floor of the fourth ventricle just cephalad to the obex was then carried out by the Grass model S4B stimulator and bipolar wire electrodes. Parameters of stimulation generally were 0.5 volts, 5/sec. (monophasic), 1 millisec. duration. During stimulation, the voltage was momentarily increased several times. Generalized clonic movements usually appeared at 0.8 to 1 volt. *Urine collection* periods were generally 20 to 30 minutes during control and stimulation. All urine secreted during stimulation was collected as one specimen. Collection periods during operative procedure were generally 1 hour. Urine sodium and potassium content was determined with Baird flame photometer using lithium as internal standard. Chloride was determined by the method of Sanderson(5). The pH was measured on Beckman pH meter.

Results. Complete data from 3 representative experiments are given in Table I. Three

TABLE I. Effect of Stimulation of Caudal Fourth Ventricle Floor on Sodium, Potassium and Chloride Excretion and Urine Volume in Dogs (3 Representative Experiments).

Dog No.			cc/min.	Na	K	Cl	Plasma Na (mEq/l)
				(μEq/min.)			
17	No intrav. fluids	Operative 9:30-10:30	.10	12	9	10	
		Control 10:30-10:55	.08	10	7	3	
		Stim. 11:17-11:47 .5 v, 5/sec.	.40	90	18	20	(Vermis resected)
		Closure 11:55-12:25	.27	50	11	14	
200	Intrav. 5% NaCl	Operative 1:10-2:10	2.4	529	41	575	
		Stim. 2:40-3:10 .5 v, 5/sec.	4.4	1090	79	1160	
		After coagulation of floor of 4th ventricle 3:17-3:47	2.1	570	44	607	
144	Intrav. 5% dextrose in water	Operative 9:45-10:45	1.25	210	26	220	Preop. 144
		Stim. 11:20-11:45 .5 v, 5/sec.	4.52	537	44	551	Before stim. 137
		After stim. 12:00-12:30	.9	41	17	58	After stim. 134
		Close 12:30-1:00	.7	12	12	15	(Vermis resected)

dogs received no intravenous fluids. In one instance, sodium and chloride excretion increased from 10 μEq/min. and 3 μEq/min. respectively before stimulation to 90 μEq/min. and 20 μEq/min. during stimulation. In the second, the sodium excretion rose from 12 to 85 μEq/min. and chloride from 8 to 101 μEq/min. during stimulation. Potassium excretion increased, but not as much as sodium or chloride.[‡] Urine volumes also rose. The effect tended to persist in the immediate post-stimulation period. In the third animal, the increase in sodium excretion was insignificant during stimulation with 0.5 volt, but increasing the parameters to 1 volt, 10 per second, resulted in a small *decrease* in sodium excretion and urine volume. During this latter period, there were continuous clonic movements of the suboccipital muscles.

One animal received a continuous intravenous infusion of 5% sodium chloride. The output of sodium, potassium and chloride doubled during stimulation of the floor of the fourth ventricle, and urine volume almost doubled (Table I). Following stimulation, the superficial caudal portion of the floor of the fourth ventricle was destroyed with electrocautery. During the next 20 minutes, excretion rates were very close to pre-stimulation values.

Continuous intravenous infusions of 5% glucose in distilled water were administered by gravity to 6 dogs. In two animals, stimulation resulted in marked increase in sodium excretion (59 to 234 μEq/min. and 210 to 537 μEq/min.) and chloride excretion (58 to 196 μEq/min. and 220 to 551 μEq/min.). There was a rise in urine volume, and very little change in potassium excretion.[§] In a third animal, sodium and chloride excretion increased slightly with stimulation at 1.5 volts, 10/sec.

In the remaining 3 dogs, the results varied. In Dog No. 101, sodium and chloride excretion increased with the electrodes in place, without electrical stimulation. The pressure of the electrodes may have acted as a stimulus. Stimulation at 0.2 volt resulted in a decrease in electrolyte excretion. There was little change at 0.5 volt, but when the stimulus was increased to 1 volt, a definite decrease in sodium and chloride output occurred. In Dogs No. 149 and 151, stimulation was accompanied by a decrease in sodium and chloride excretion, with an increase following stimulation in No. 151.

Two additional experiments were carried out, but are not listed. In 2 dogs, the orbital surface and the convexity of the frontal lobe were stimulated at varying voltages. One dog received 5% dextrose in distilled water, the other 5% sodium chloride. No definite change in urine volume or electrolyte excretion was noted.

Discussion. The bipolar method of stimulation used in these studies probably results in stimulation of a relatively large cross-sectional area of the brain stem at the level stimulated, depending on voltage. Thus at 0.7 to 1 volt, clonic movements of the extremities occurred, presumably from stimulation of the pyramidal tracts. It was noted, however, that voltage threshold for clonic movements varied from one animal to the next, suggesting that at the voltage selected (0.5 volt), the spread of the stimulus varied somewhat among the animals used. This may explain in part the variability of the results.

The data obtained, however, suggest that an influence may be exerted by the brain stem on renal excretion of electrolytes, particularly sodium and chloride. In 5 dogs, stimulation of the caudal portion of the floor of the fourth ventricle at 0.5 volt, 5 cycles per second, resulted in increased excretion of sodium and chloride. A smaller increase in potassium excretion also occurred in these dogs. Slight rises of questionable significance occurred in 3 dogs, and there was some decrease in excretion rate in 2 dogs. Increasing the stimulus strength to 1 volt (and thereby presumably increasing the spread of the stimulus) in 2 dogs resulted in a decrease in excretion rates.

Previous studies of the effects of brain stimulation(6), increased intracranial pressure(7,8) and splanchnicotomy(9-11) suggest that the nervous system may influence renal electrolyte excretion. The author is not aware of studies similar to the present one.

[‡] Concentration of sodium and chloride in urine (mEq./l.) also increased during stimulation, while concentration of potassium decreased.

[§] In Dog No. 144, see Table, the serum sodium had fallen from 144 mEq./l. to 137 mEq./l. prior to stimulation.

These results are preliminary. Further studies are planned.

Summary. In anesthetized dogs, bipolar stimulation (0.5 volt, 5/sec.) of the caudal portion of the floor of the fourth ventricle resulted in increased renal excretion of sodium and chloride, and a smaller increase in potassium excretion in 5 instances, slight changes in 3, and decreased excretion in 2. In 2 dogs, increasing the stimulus to 1 volt resulted in a decreased excretion rate of these electrolytes.

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Identification of Cystine Calculi in Mink. (22327)

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Leoschke, Zikria and Elvehjem(1) in quantitative analyses of 31 urinary calculi from mink revealed the main constituent to be magnesium ammonium phosphate, accompanied by small amounts of calcium phosphate and magnesium phosphate. Sompolinsky(2), on the basis of qualitative analyses of 26 stones classified 12 as pure magnesium ammonium phosphate, 13 as magnesium ammonium phosphate plus calcium and 1 as magnesium ammonium phosphate plus calcium and urate.

Observations on bladder stones from mink submitted to the Veterinary Diagnostic Laboratory at Oregon State College have generally indicated an inorganic-type structure. Recently, however, a male mink was presented whose bladder contained 140 small creamy-white colored, smooth, irregularly-shaped calculi and these when submitted to normal ashing procedures proved to be wholly organic matter. Total oven dry weight of the calculi was 1.023 g. The appearance and size of these calculi are indicated in Fig. 1.

Observations. Qualitative analyses of these stones were carried out in the sequence proposed by Hawk, Oser and Summerson(3). These tests indicated strongly the presence of cystine.

Further identification was made by means of Sullivan's reaction which is reported to be specific for cystine(4). A few mg of powdered calculus were taken up in 5 ml of approximately 0.1 N HCl and 1 ml each of 1% NaCN in 0.8 N NaOH and 0.5% aqueous 1, 2-napthoquinone-4-sodium sulfonate were added. These were followed by the addition of 5 ml 15% Na_2SO_3 in 0.5 N NaOH and the mixture was allowed to stand 30 minutes. On

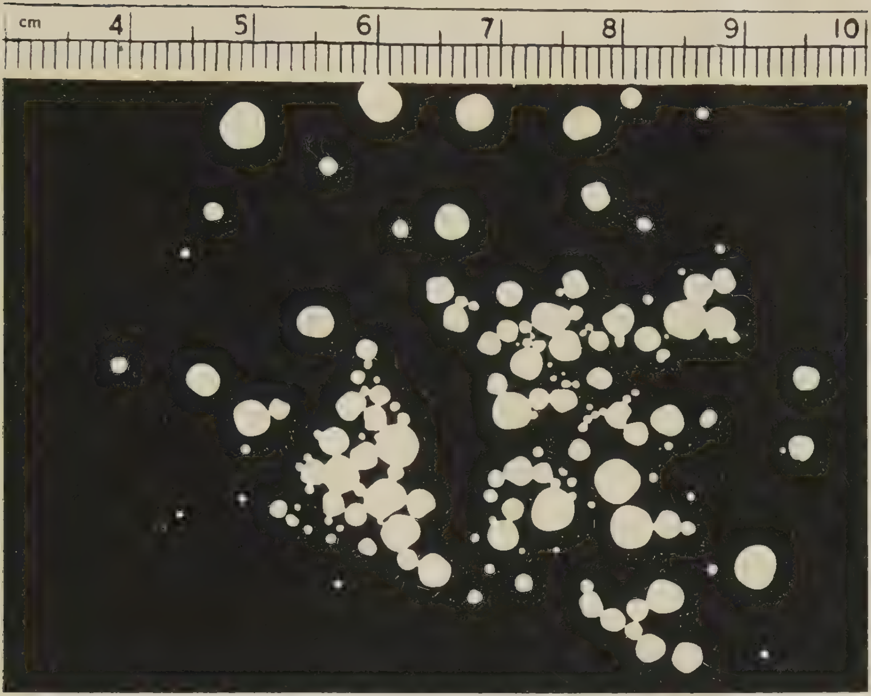

FIG. 1. Cystine calculi from the bladder of a mink.

addition of 1 ml of 2% aqueous sodium dithionite ($Na_2S_2O_4$) a clear red color developed. This test was checked against pure DL cystine with positive results.

Finally, a quantitative test for cystine was carried out upon the dried, ground calculus material, following the method of Lugg(5), using a phosphotungstic acid reagent prepared according to directions of Folin and Marenzi (6). This test revealed the stones to be essentially completely (99%) cystine.

Discussion. Occurrence of cystine calculi in the bladder of dogs, first noted by Lassaigne in 1823(7), has since been reported by several workers(8-11), however, no reference to this type of stone in mink has been found in the literature. It is interesting that the case cited herein involved a male mink, since studies with humans and with dogs(8) have indicated that cystinuria is mainly confined to the male sex, however, this may be due to the constriction of the urethra in the male, where even small stones of the cystine type might cause stoppage.

The diet fed on the ranch where the cystine calculi occurred was not unusual, consisting of approximately 55% Pacific coast fish, 25% tripe, 10% liver and 10% commercially prepared cereal mixture. Since rations fed ranch mink are customarily high in animal protein, however, there may be some dietary predisposition towards high cystine excretion.

Summary. One hundred forty small (0.3 to 3.1 mm dia.) calculi were obtained from the bladder of a male mink on autopsy. Both qualitative and quantitative analyses of these stones indicated that they were almost entirely composed of cystine.

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Effect of Lyophilization and Chloroform Extraction on Electrophoretic Migration of Serum Lipoproteins.* (22328)

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It has been shown that when serum from normal human subjects was lyophilized and extracted with cold chloroform for 3 hours only a small percentage of the total serum cholesterol was extracted(1). This fraction has been designated the "readily extractable cholesterol"—REC. The value for the REC fraction was shown to be 40 mg % or less in 98% of 687 subjects between the ages of 17-33 years, but the percentage of subjects with extraction values above 40 mg % was found to increase up to about the fifth decade(2,3). This was especially marked in the executive and professional groups studied. Subjects with coronary infarction or with atherosclerotic heart disease showed a still higher percentage with elevated extractions.

In an attempt to obtain information concerning the lipoprotein fractions from which the cholesterol was extracted, paper electrophoretic studies were carried out on a) original serum, b) reconstituted lyophilized serum, and c) reconstituted chloroform-extracted, lyophilized serum. Lyophilized sera were reconstituted by the addition of an amount of water equal to the original volume of serum, namely, 0.5 ml. Since the volume occupied by the serum solids was ignored, the concentration of the various substances in these solutions was slightly less than in the original serum.

Method. A Kelab paper electrophoresis apparatus was employed using Whatman #3 filter paper strips (4.6 x 35 cm) with a barbital-sodium acetate buffer of pH 8.6 and ionic strength .05. Three paper strips separated by plastic strips were used in each of the 2 compartments of the apparatus, thus making a total of 6 strips for each run. To each of the strips at a line 12 cm from the cathode end, 0.05 ml of the test solution was applied. The electrophoresis was run for 15 hours at 5°C unless otherwise stated, with 1.2 ma per strip and a potential of approximately 210 volts. The strips were removed from the apparatus at the end of the electrophoretic procedure and processed as follows: 2 strips were dried at 100°C for 45 minutes, then stained for protein with bromphenol blue according to the method of Köiw, *et al.*(4); 1 strip was air dried and stained for lipids with Sudan black-B; and the remaining 3 strips were kept moist by suspending them in a 1000 ml cylinder containing about half an inch of water and covered with an inverted Petri dish to reduce evaporation. As soon as the position of the proteins along the stained strips was determined, which seldom exceeded 90 minutes from the time the strips were suspended in the cylinder, the wet strips were cut as indicated below, the sections transferred to 25 ml glass-stoppered Erlenmeyer flasks, and approximately 17 ml of a 1:1 mixture of ethyl alcohol and ethyl ether added to each flask. Eight sections were prepared: 1) the albumin area which also included the alpha$_1$-globulins, 2) the area between the al-

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bumin + alpha$_1$-globulins and alpha$_2$-globulins, 3) the alpha$_2$-globulins, with the cut being made half way between the alpha$_2$- and beta-globulins, 4) the beta-globulins, 5) a section between the beta-globulins and 5 mm short of the starting point, 6) a section including the area 5 mm on either side of the starting point which usually included some of the gamma-globulins, 7) the remainder of the gamma-globulins, and 8) a 10 mm section towards the end of the strip to serve as a blank. The amount of cholesterol extracted from these sections was determined using a procedure described previously(5).

Results. Effects of lyophilization. The experimental results are shown in Table I. The values for sections 1 and 2 as well as for sections 6 and 7 have been combined. It will be seen that lyophilization invariably increased the amount of cholesterol remaining around the starting point while decreasing that found in the area of the beta-globulins. The effect was most noticeable in those sera with markedly elevated REC values. The REC concentration seems to be of greater significance in this regard than the total cholesterol concentration since, on the whole, the percentage of the total cholesterol which remained around the starting point in the case of the lyophilized sera was greater in Group 3 than in Groups 1 and 2. Each of these groups showed normal total cholesterol concentration but the REC value was quite elevated in the case of Group 3. The percentage of the total cholesterol present in the albumin + alpha$_1$-globulin area was distinctly greater in those subjects with normal REC values than in those with markedly elevated REC concentrations. The rate of movement of the cholesterol associated with albumin and alpha$_1$-globulins was usually not affected by lyophilization. Actually only one of the 21 sera tested showed a definite drop in the cholesterol content of the albumin + alpha$_1$-globulin area following lyophilization. Apparently lyophilization affects primarily the migration of the cholesterol normally present in the general area of the beta-globulins.

Extractability of the lyophilized serum by cold chloroform: Experimental data on the effect of chloroform extraction on the cholesterol distribution are shown in Table II. The values from sections 1-3 have been combined and also those of sections 6 and 7. The sera studied fall into 3 main groups: a) those with normal REC values, b) those with fairly elevated REC values and c) those from which the cold chloroform extracted a large percentage of the total cholesterol. It will be seen that in those with normal REC concentrations most of the cholesterol extracted came from around the starting point. When the REC concentration was very high most of the cholesterol between the beta-globulins and starting point was removed. In 2 cases with extremely high extraction values, no demonstrable cholesterol was left in this area.

Discussion. Previous work has shown that lyophilization, even when followed by cold chloroform extraction, caused no demonstrable change in the migration of the main serum proteins as determined by the bromphenol blue technic(6). The beta-lipoproteins apparently constitute such a small portion of the beta-globulins that a change in their rate of migration is not demonstrated by the bromphenol blue technic. Hunter(7) found no bromphenol blue staining in the area giving the strongest staining with osmium tetraoxide vapors. The ratio of the cholesterol present in the albumin + alpha-globulin area to that present in the remainder of the strip in the 6 normal sera was the same as found by Oliver and Boyd(8) in normal subjects, namely, 28:72. The ratio for all subjects with elevated REC values was invariably less than this.

The fact that the cold chloroform usually removed little cholesterol from the albumin + alpha$_1$- and alpha$_2$-globulin areas can probably be explained by the greater stability of alpha-lipoproteins. Gurd, *et al.*(9) showed that alpha-lipoproteins are stable to lyophilization but purified beta-lipoproteins are not. Since the lyophilized serum in all cases still contained lipoproteins which migrated in the general area of the beta-globulins it would appear that some of the beta-lipoproteins are not denatured by lyophilization. The higher the REC value the greater was the percentage of total cholesterol which remained around the starting point. Swahn(10) has shown

TABLE I. Effect of Lyophilization on Electrophoretic Migration of Serum Cholesterol.

Specimen	Group & No. of subjects	% of total cholesterol in sections 1, 2	3	4	5	6, 7	REC* (range), mg %	Total chol. (range), mg %
Sera from young normal male subjects	1 (6)	21.7 (16.0–31.4)†	6.1 (4.4– 8.2)	37.8 (26.8–52.6)	29.3 (14.6–40.8)	5.0 (1.1–10.3)	16– 35	179–275
Same lyophilized		20.3 (16.2–22.8)	5.7 (2.0–11.6)	20.1 (6.2–27.6)	35.5 (24.5–43.6)	18.3 (13.6–25.1)		
Sera from patients with normal REC & total chol.	2 (7)	14.7 (10.8–14.6)	8.2 (2.8–17.0)	48.1 (40.4–51.7)	22.4 (11.8–33.3)	6.7 (4.7–10.3)	15– 35	141–291
Same lyophilized		13.8 (9.0–20.9)	6.4 (5.8– 7.0)	22.8 (14.1–29.1)	30.9 (19.4–39.7)	26.0 (17.5–33.1)		
Sera from patients with elevated REC & normal total chol.	3 (3)	8.3 (5.9– 9.8)	8.3 (2.7–14.1)	52.4 (51.5–54.1)	25.7 (23.7–28.7)	5.3 (4.7– 5.8)	141–180	256–269
Same lyophilized		8.6 (7.9– 9.2)	4.9 (4.0– 6.5)	22.2 (19.2–24.2)	31.9 (30.8–33.1)	32.3 (29.1–35.0)		
Sera from patients with elevated REC & total chol.	4 (4)	10.7 (7.7–11.2)	3.6 (2.0– 4.8)	46.5 (37.5–56.5)	32.6 (26.8–41.1)	6.7 (3.4–11.2)	107–240	353–430
Same lyophilized		9.6 (8.8–10.7)	3.2 (2.6– 3.7)	11.4 (7.7–16.0)	38.5 (33.8–43.5)	37.3 (36.9–40.7)		
Serum from patient with markedly elevated REC & total chol.	5 (1)	3.5	10.6	64.5	15.9	5.5	670	670
Same lyophilized		4.6	2.8	8.1	12.3	72.1		

* Readily extractable cholesterol—that which cold chloroform extracts from lyophilized serum. † Range in parentheses.

TABLE II. Cholesterol Distribution after Electrophoresis of Lyophilized Sera before and after Chloroform Extraction.

Sample source	Group & No. of subjects	Sections 1, 2, 3 (mg %)	4	5	6, 7	Sum of section values	REC* (range)
Normal subjects	1 (5)	50 (30–66)‡	36 (14– 62)	72 (44–101)	39 (25– 61)	197	16– 35
Residue†		49 (37–64)	30 (18– 37)	72 (57– 88)	22 (10– 34)	173	
Patients with normal REC & total chol.	2 (7)	45 (33–55)	51 (32– 74)	71 (41–109)	58 (29– 76)	225	20– 36
Residue		43 (22–50)	45 (27– 56)	61 (39– 92)	41 (22– 67)	190	
Patients with moderately elevated REC	3 (7)	45 (27–61)	62 (29–101)	113 (85–151)	94 (66–130)	314	66–141
Residue		48 (33–69)	52 (38– 74)	76 (53– 89)	55 (26– 80)	231	
Patients with markedly elevated REC & total chol.	4 (5)	55 (42–90)	74 (48–117)	135 (96–185)	250 (141–451)	514	240–833
Residue		36 (26–47)	25 (7– 55)	26 (5– 53)	17 (0– 51)	104	

* Readily extractable cholesterol—that which cold chloroform extracts from lyophilized serum. † Lyophilized sera after extraction with cold chloroform. ‡ Range in parentheses.

that in paper electrophoresis, chylomicrons remained around the starting point while lipomicrons migrated with the beta-globulins. Since lipomicrons would probably be denatured by lyophilization it is possible that the increase in the cholesterol remaining around the starting point in the lyophilized sera may represent denatured lipomicrons.

The results which have been presented suggest that the cholesterol which is extracted by cold chloroform from lyophilized serum is primarily that present as a constituent of lipo- and chylomicrons. Additional work, however, is necessary to establish this. It is interesting that Albrink, Man and Peters(11) found that the neutral fat which was removed by centrifugation at 18,000 rpm for 1 hour from clear and lactescent sera having total cholesterol values above 300 mg per 100 ml varied from 16.8 to 478 mEq per liter while the amount remaining in the subnatant varied only from 10 to 34 mEq per liter. They assume that the lipids which rose to the surface ranged from 0.1 μ in diameter, the size just sufficient to scatter light(12) to 1 μ, the size of larger chylomicrons. Any cholesterol associated with this material undoubtedly would be included in our REC fraction.

Summary. Lyophilization of serum has been shown markedly to retard electrophoretic migration of some of the cholesterol normally associated with the beta-globulin area but to exert little effect on the cholesterol associated with the albumin + alpha$_1$-globulins. The cholesterol which is extracted by cold chloroform from lyophilized serum comes primarily from that fraction normally found in the beta-globulin area or which remained around the starting point. It is suggested that the cholesterol thus removed represents primarily cholesterol which is associated with lipo- and chylomicrons.

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Coagulation of Salivary Mucoid by Freezing and Thawing of Saliva.* (22329)

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Salivary mucoid,† especially when freshly obtained, may be readily precipitated in the form of a coagulum by the addition of dilute acid to the mucoid-containing fluid(2-4). This characteristic has been used to identify and to prepare mucoid of salivary gland origin(2-5).

It has now been observed that freezing of chemically untreated mucoid-containing salivary secretions for varied periods of time re-

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† The terminology suggested by Meyer(1) is used throughout this report.

sults in the formation of a persisting coagulum when the secretion is thawed. This coagulum, or clot, generally resembles in appearance the mucin clot formed in response to the addition of acid. Evidence from viscosity measurements, response to dilute acetic acid, and analyses of "sialic acid" indicates that the coagulum formed after freezing and thawing is derived from the salivary mucoid.

Methods. Total saliva and the separate salivary secretions were collected by procedures described previously(6,7). *Chemical Methods.* The mucin clot test for the presence of polymerized mucoid was performed by adding 2 volumes of 3% acetic acid to one volume of saliva. "Sialic acid", which appears to represent the principal constituent of the carbohydrate moiety of the mucoid(4), was detected by the direct Ehrlich reaction according to the method of Werner and Odin (8). Since for this investigation only the direction of change in "sialic acid" concentration resulting from removal of the clot following freeze-thaw treatment is of importance, only relative concentrations of "sialic acid" (in terms of optical densities measured at 5650 Å in the Coleman spectrophotometer) are indicated. For amylase determinations, salivary samples were diluted 1:1000 with .025 *N* sodium chloride buffered to pH 6.9 with .01 *M* sodium phosphate. The reaction mixture consisted of 1.0 ml buffered .025 *N* sodium chloride, 1.0 ml stock soluble starch solution(9), and 0.5 ml diluted saliva. Digestion was for 15 minutes at 37.0°C. Enzyme activity is expressed in terms of milligrams of reducing sugar x 10 formed from 10 mg of substrate under these conditions. The method of Myers, Free, and Rosinski(9) was used for determination of reducing sugar. *Physical methods.* Freezing of saliva samples was accomplished either by immersion of a pyrex container in a dry ice-acetone mixture at −60 to −70°C or by placing at −10°C in the freezing compartment of a refrigerator. In each case the method and duration of freezing are stated. Thawing took place at room temperature (22-25°C) in all cases. Saliva it not a true Newtonian fluid; consequently the rate of flow of salivary fluid through a narrow bore tube in response to a difference in pressure may, to some degree, reflect the influence of physical characteristics other than viscosity. Nonetheless, it is generally true that this resistance to flow results principally from the presence of polymerized salivary mucoid and decreases when the mucoid is depolymerized(10,11) or removed as by acid clotting. With the reservations imposed by the nature of the salivary fluid clearly recognized, therefore, viscosity measurements may be used to reflect grossly the concentration of polymerized salivary mucoid(11,12). In this investigation, viscosity measurements were made using graduated capillary pipettes held vertical in matched test tubes containing 2 ml of salivary fluid. The pipettes were calibrated by observing emptying times for a 40% sucrose solution.

Results. The appearance of a persisting clot in fresh samples of frozen and thawed, but otherwise untreated, total mixed, submaxillary and sublingual salivas has been observed using more than 200 secretion samples from at least 40 individual subjects. The clot appears in the mucoid-containing secretions within 2 minutes if dry ice is employed for the freezing stage. That the clot formed after thawing is derived from the mucoid is indicated by observations resulting from 3 types of experiments. In the first type, dilute acetic acid was added to an untreated aliquot of a submaxillary secretion sample and to the supernatant fluid of a frozen-thawed aliquot at the same time after collection of the sample. Fig. 1 shows clearly the mucin clot which formed in the control and that no clot or precipitate appeared in the supernatant of the frozen-thawed aliquot.

The viscosity of a sample of submaxillary saliva standing at room temperature (25°C) may decrease by 50% within 40 minutes after collection (Table I). This effect has been attributed to nonenzymatic(10) and enzymatic(13) depolymerization processes. Freezing and thawing of an aliquot of the submaxillary saliva sample followed by removal of the resulting clot leads to an even more marked reduction of viscosity than is observed for the control aliquot within the same time

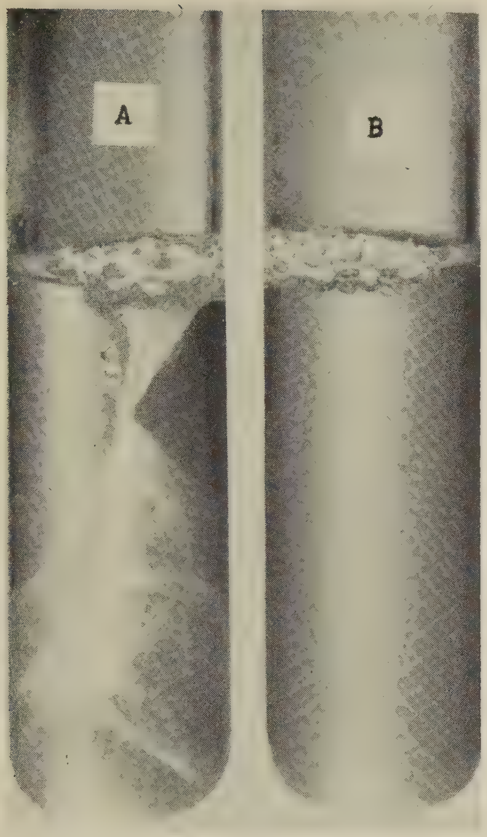

FIG. 1. Effect of addition of dilute acetic acid to an untreated aliquot (A) and to supernatant fluid of a frozen-thawed aliquot (B) of a submaxillary secretion sample 40 min. after collection. Note absence of acid-induced clot in (B), indicating decrease in polymerized mucoid in supernatant after freezing at –60°C for 2 min. and subsequent thawing at 25°C.

period after collection and indicates significant added loss of polymerized mucoid from solution as a result of the freeze-thaw treatment (Table I).

TABLE I. Effect of Freezing and Thawing on Viscosity of Submaxillary Secretion Samples.

	Sample I	Sample II
	Centipoises	
Initial viscosity	9.2	13.4
Final viscosity* (no freeze-thaw)	6.7	6.6
Final viscosity* (after freeze-thaw†)	2.4	1.8

* Final viscosity was determined 40 min. after initial viscosity determination. Except for period of freezing (where indicated), aliquots were kept at 25°C.

† Frozen by immersion in dry ice-acetone mixture at –60°C for approximately 2 min., thawed at 25°C, centrifuged, and clot discarded.

A third line of investigation which indicates that the clot which appears following thawing is derived from mucoid consists of comparing "sialic acid" values of sample aliquots which are untreated and those from which the clot appearing after freezing and thawing has been removed. "Sialic acid" is a major constituent of submaxillary mucoid and represents 25-30% of its dry weight(4). As shown by the data given in Table II, a marked decrease in "sialic acid" occurs in those aliquots submitted to freezing and thawing followed by removal of the resulting clot (Table II).

It may be noted also that clot formation after freezing and thawing is not observed with parotid secretion samples. The "sialic acid" analysis for a parotid secretion sample noted in Table II suggests the absence of "sialic acid" from this secretion. These observations are consistent and further indicate that the clot resulting from freezing and thawing of saliva samples is derived from the salivary mucoid.

TABLE II. Effect of Freezing and Thawing on Relative Concentration of Sialic Acid in Salivary Secretion Fluids.

Subject	Sample	Aliquot I (untreated)*	Aliquot II (frozen-thawed)
		Optical density × 10^2	
HF†	Submaxillary	14	5
	"	14	5
FB‡	Parotid	0	
	Sublingual	34	
	Submaxillary	17	7
LS§	"	15	3
LL§	"	43	7

* Untreated aliquots kept at 25°C before analysis.

† Aliquots I and II were submitted to analysis simultaneously 40 min. after collection of sample. Before analysis, Aliquots II were frozen 2 min. in dry ice-acetone mixture, thawed at room temperature, centrifuged and the clots discarded.

‡ Aliquots I were submitted to analysis within 30 min. after sample collection. Aliquot II (submaxillary sample) was submitted to analysis after freezing for 1 hr at –10°C and thawing at room temperature.

§ Aliquot I was submitted to analysis immediately after collection of the sample. Aliquot II was frozen 24 hr at –10°C and thawed at room temperature before analysis.

The extent to which the concentration of substances, other than mucoid, may be modified in the salivary fluid by inclusion in the coagulum has been estimated at this time only for the protein enzyme amylase. The data in Table III indicate that the amylase concentration of the salivary fluid is not appreciably modified either by the freezing and thawing procedure or by removal of the clot resulting from this procedure (Table III).

TABLE III. Effect of Freezing and Thawing of Saliva Samples on Their Amylase Activity, 5 subjects.

	Amylase activity*			
	Unfrozen aliquot		Frozen-thawed aliquot	
Secretion	Immed. after collection	After 24 hr at 4°C	Clot removed	Clot resuspended
Submaxillary	2.2	2.2	2.3	2.5
Parotid	7.9	7.9	7.9	
Submaxillary	6.8	6.4	6.2	5.4
Parotid	13.9	12.8	12.3	
Submaxillary			3.8	4.7
Total mixed			2.7	2.7
Idem			2.8	3.1

* Conditions: Amylase activity is expressed as mg reducing sugar × 10 formed at 37°C from 10 mg of starch by the action of .5 ml 1:1000 saliva. Freezing (where indicated) was for 24 hr at –10°C in refrigerator; thawing occurred at room temperature (22-25°C).

Discussion. Gore has reported that stimulated total or mandibular (mixed submaxillary-sublingual) saliva shows some opacity at collection and that on standing at 37°C for one hour appreciable precipitate, presumably of mucoid material, appears and settles out (14-16). This effect may possibly be ascribed to the action of a contaminating mucinase (12,13). The separate submaxillary secretion obtained for the experiments reported here not only has shown no opacity on collection but generally shows only slight turbidity even after standing for two hours at 25 or 37°C. For the clear delineation of the freeze-thaw effect, therefore, separate submaxillary secretion has generally been used.

The observation that a persisting clot appears after thawing of frozen fresh, mucoid-containing saliva samples and the experimental data which show clearly that this clot is derived from salivary mucoid may have application from several points of view. First, it is customary and convenient to freeze tissue or fluid samples for storage before analysis. In the case of saliva samples, care must be taken to determine that such treatment does not alter the composition relative to the analysis in question. Second, freezing and thawing may provide a convenient method for reducing the viscosity of the mucus gland secretions especially in cases where this does not alter the concentration of other components. In addition, the freeze-thaw method may prove useful as a gentle procedure for the preparation of mucoid. This method has been used successfully for the preparation of fibrinogen of extreme purity(17). The purity of salivary mucoid separated by freezing and thawing has not been determined, particularly because of difficulties involved in establishing adequate criteria of purity(1). Finally, a difference in physico-chemical properties of salivary mucoid and other mucus secretions is indicated, since Ronkin(18) has reported no change in properties after thawing frozen hyperbranchial mucus secretion from the marine snail, *Busycon*.

Summary. 1. Freezing of chemically untreated mucoid-containing salivary secretions results in the formation of a persisting clot when the secretion is thawed. This clot resembles in appearance the mucin clot formed in response to the addition of acid. 2. Evidence from viscosity measurements, response to addition of weak acid, and "sialic acid" analyses indicates that the coagulum formed after freezing and thawing is derived from the salivary mucoid. 3. It is suggested that this effect be taken into account if storage of saliva samples in a frozen state is considered.

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Serum Glutamic Pyruvic Transaminase in Cardiac and Hepatic Disease.* (22330)

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Characteristic alterations of serum glutamic oxaloacetic transaminase (SGO-T) activity have been shown to be useful in assessing the presence and degree of heart muscle cell and hepatocellular damage(1,2). The activities of glutamic oxaloacetic (GO-T) transaminase and of glutamic pyruvic transaminase (GP-T) in different tissue homogenates are compared in Table I. The activity of GP-T was found to be relatively greater in liver than other tissues as compared with GO-T activity. This observation suggested that serum glutamic pyruvic transaminase (SGP-T) might be a more specific index of liver cell damage than SGO-T because of its selective concentration in liver tissue. Furthermore, since the cardiac tissue activity of GP-T is low, myocardial necrosis might not be associated with significant alterations of SGP-T activity, thus providing a more specific serum enzyme indicator of hepatocellular damage.

Method of study. The SGP-T activity was measured spectrophotometrically. The transamination reaction (Reaction 1) is coupled to the reduction of pyruvate to lactate by reduced diphosphopyridinenucleotide (DPNH), in the presence of added excess of purified lactic dehydrogenase (Reaction 2).

$$\begin{array}{l} \alpha\text{-keto glutarate} \\ \quad + \text{l-alanine} \end{array} \xrightarrow{\text{SGP-T}} \begin{array}{l} \text{glutamate} \\ \quad + \text{pyruvate} \end{array} \quad \text{(Reaction 1)}$$

$$\begin{array}{l} \text{pyruvate} \\ + \text{DPNH} \end{array} \xrightarrow[\text{dehydrogenase}]{\text{lactic}} \begin{array}{l} \text{lactate} \\ + \text{DPN} \end{array} \quad (\quad " \quad 2)$$

Oxidation of DPNH, and thereby the transamination reaction, is followed by measuring the decrease in light absorption at wavelength 340 mμ at which reduced diphosphopyridinenucleotide has an absorption peak. The *reagents* include: alanine, α-keto-glutaric acid, reduced diphosphopyridinenucleotide, and purified lactic dehydrogenase which can be obtained commercially. From 0.1 to 1.0 ml of serum, 1.0 ml of 0.1 M phosphate buffer (pH 7.4), 0.5 ml of 0.2 M l-alanine in buffer (pH 7.4), 0.2 ml of DPNH (1 mg/ml) and 0.1 ml of a solution of purified lactic dehydrogenase (8000 units/1.0 ml) were mixed and brought to final volume of 2.8 ml in a cuvette having a 1 cm light path. The blank contained all reactants listed except DPNH. After 15 minutes, 0.2 ml of 0.1 M α-keto-glutarate in buffer, pH 7.4, was added. The optical density at wavelength 340 mμ was

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TABLE I. Comparison of Glutamic Oxaloacetic Transaminase and Glutamic Pyruvic Transaminase in Normal Adult Tissue Homogenates.

Tissue	GO-T	GP-T
	Units/g wet tissue (× 1000)	
Heart	156	7.1
Liver	142	44
Skeletal muscle	99	4.8
Kidney	91	19
Pancreas	28	2
Spleen	14	1.2
Lung	10	.7
Serum	.02	.016

followed for 5 minutes and the rate of decrease in optical density taken as measure of the glutamic pyruvic transaminase activity of the serum. The reaction was followed in a Beckman DU spectrophotometer at room temperature (23°C). Serum activity is expressed in units/ml/minute. One unit equals a decrease in optical density of 0.001 mμ under the conditions described.

Results. Glutamic pyruvic transaminase was measured at 23°C in sera of 260 normal humans and the activities had a mean value of 16 ± 9 units/ml/minute. When the mean value of SGP-T activity is converted to micromoles/ml/hour the values fall within the range of 0.23 to 0.82 micromoles/ml/hour found by quantitative paper chromatographic assay previously reported(3).

Unless hepatic disease is simultaneously present, SGP-T appears not to be significantly altered above the normal range of activity by infectious, neoplastic, reactive, degenerative, congenital or allergic disease states. The SGP-T fails to increase significantly following necrosis resulting from acute myocardial infarction. This is probably accounted for by the fact that GP-T activity is low in heart muscle in contrast to the high activity of GO-T in cardiac tissue. Table II lists the SGO-T and SGP-T activities observed in patients with acute and chronic hepatic and cardiac disease states. From these observations and studies of 40 patients with various acute hepatic diseases it appears that acute hepatitis is associated with a rise in SGP-T activity of from 2 to 1000 times normal. The peak rise during the course of clinical hepatitis is roughly proportional to the severity of the disease process. In acute hepatitis the SGP-T activity was greater than that of SGO-T although the curves are closely parallel. The same similarity of SGP-T and SGO-T concentration was seen in patients with extrahepatic biliary obstruction. Cirrhosis in relapse is associated with increased SGO-T and SGP-T activity but the SGO-T activity is usually greater than the changes in SGP-T. Infiltration of liver by metastatic cancer, leukemic or lymphomatous disease may alter the SGO-T but has not been seen to cause elevation of the SGP-T above the normal range. The SGP-T activity has not been found to increase significantly following acute myocardial infarction in 8 patients, nor in 15 patients with other forms of heart disease. The increase in SGO-T activity in acute hepatic disease has been suggested to result from the release of liver-cell glutamic oxaloacetic transaminase into the blood stream from damaged liver cells(4,5). The presence in hepatic tissue homogenates of considerably less glutamic pyruvic transaminase activity than glutamic oxaloacetic transaminase suggests that the relatively greater increment of SGP-T activity in acute liver disease could not be solely due to release of enzyme from necrotic hepatic tissue. Variations in the release, destruction or excretion of the two enzymes or an unknown metabolic aberration are probably important contributory mechanisms and have yet to be clarified. The increase in SGO-

TABLE II. Comparison of SGO-T and SGP-T in 11 Patients with Hepatic and Cardiac Disease.

Diagnosis		SGO-T	SGP-T
		Units/ml	
Normal individuals	Range	8-40	5-35
	Mean	20	16
Acute infectious hepatitis		330	720
Idem		1380	1800
Acute infectious mononucleosis hepatitis		82	120
Acute extrahepatic obstructive jaundice		108	224
Cirrhosis		134	70
Hodgkins disease of liver		43	16
Acquired hemolytic jaundice		35	24
Acute transmural myocardial infarction		61	18
Idem		228	32
Acute subendocardial infarction		56	15
Angina pectoris		35	20

T following acute myocardial infarction would appear to be primarily due to release of enzyme from necrotic cardiac muscle(6). The failure of significant elevation of SGP-T is probably due to the relatively small and therefore inconsequential amount of glutamic pyruvic transaminase escaping from damaged heart cells which when diluted in the extracellular compartment would fail appreciably to increase the SGP-T.

Summary. The measurement of SGP-T alterations has been found to be a useful tool in the diagnosis and study of acute hepatic disease and appears to be more sensitive than SGO-T in depicting acute hepatocellular damage. SGP-T unlike SGO-T has the added advantage of not being appreciably altered by acute cardiac necrosis. These preliminary conclusions require further study for confirmation.

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Vitamin A Content of the Livers of Suckling Pigs.* (22331)

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The liver of the normal newly born rat was reported by Guerrant(1) to contain very little vit. A but that the concentration more than doubled during the first week of postnatal life and then remained at a relatively constant level during the remainder of the nursing period. Braude *et al.*(2) reported liver vit. A reserves to be low in the pig at birth with the amount still decreasing during suckling if the sows were fed a vit. A deficient ration.

This paper reports the levels of vit. A found in the livers of pigs which suckled their dams for varying lengths of time.

Methods. This investigation was conducted in the summer. Thirty-five baby pigs from 6 cross-bred litters were used. One pig from each litter was sacrificed at weekly intervals until the litter was exhausted, the first group of 6 being sacrificed before they were 24 hours old. The mothers of the pigs were fed well-fortified rations containing sun-cured alfalfa hay throughout gestation and lactation. The pigs' only feed was the milk of their dam.

TABLE I. Average Vitamin A Content of Livers of Suckling Pigs.

Age at sacrifice, days	No. of pigs	Body wt, lb	Liver wt, g	Liver fat, %	Liver vit. A	
					Per g (fresh), μg	Total, μg
1	6	3.2 ± .5*	38 ± 7.9	3.4 ± .4	15.1 ± 2.6	570 ± 152
7	6	5.5 ± .9	76 ± 7.5	3.3 ± .5	30.6 ± 9.3	2281 ± 456
14	6	8.0 ± 1.9	104 ± 27.7	3.3 ± .5	26.4 ± 5.7	2689 ± 528
21	5	10.2 ± .6	135 ± 19.7	3.6 ± .6	24.4 ± 9.0	3184 ± 640
28	5	13.8 ± 3.9	179 ± 45.5	3.6 ± .4	21.7 ± 5.5	3889 ± 1622
35	5	18.5 ± 2.8	235 ± 49.4	3.3 ± .3	19.3 ± 3.4	4539 ± 975
42	2	16.9 ± 1.2	—	3.8 ± .8	17.4 ± 2.0	—

* Stand. dev. of single observation.

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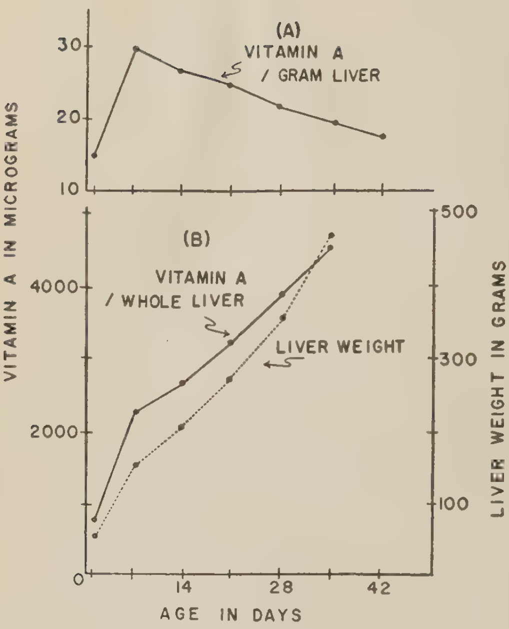

FIG. 1. Average vit. A contents in liver of suckling pigs.

The sows were fed separately from their litters and solid food was never found in the stomachs of the sacrificed pigs. The number of livers tested at each age is shown in Table I. Livers were weighed and frozen immediately after sacrifice of the pigs. Fat was extracted with ether. Vit. A was determined by the method of Gallup and Hoefer(3).

Results. Summaries of body weights, liver weights, percent fat in liver and liver vit. A according to age of pigs are presented in Table I. At one day of age, the concentration of vit. A was 15.1 μg per g of liver, while the hepatic stores for the grown-up pigs, as determined in the same season, was several times higher. The relatively low reserve of this vitamin in the liver at birth agrees well with other species(4). By the 7th day this low reserve of vit. A was doubled per unit weight and increased 4 times (570 to 2881 μg) for the whole liver while the average liver weight increased from 38 to 76 g. This increase in vit. A in the pig seems to be accounted for by the finding of Braude *et al.* (5) that sow colostrum is richer than normal milk in this vitamin.

After reaching a peak at 7 days, the concentration of vit. A in the liver decreased gradually and uniformly from about 31 to 19 μg per g of liver at 35 days after parturition (Fig. 1A). The livers grew in size with age, however, so that the total liver vit. A made a relatively steady increase from birth until the pigs were 35 days old reaching a maximum of about 4500 μg (Fig. 1B).

Summary. Livers of 35 pigs from 6 different litters were examined for vit. A content. The liver vit. A concentration increased from 15 μg at 1 day to 31 μg per g at 7 days after parturition. The average total liver vit. A increased rather uniformly from 570 μg at birth to 4539 μg at 35 days after birth.

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Propagation of Frog Renal Carcinoma in Embryonated Hens' Eggs.* (22332)

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The frog renal carcinoma (Lucké) is readily propagated in tissue culture and by intraocular implantation in frogs; homo- and heterotransplants can be made(1). It can also be grown by implantation of tadpoles (2). In the salamander the character of the tumor is reported to be altered(3). Propagation of the tumor in embryonated hens' eggs was attempted in the present study.

Materials and methods. The tumors used were from experimental frogs kindly sent by Dr. B. Lucké from Philadelphia. Naturally occurring renal tumors were also obtained from *R. pipiens* received from J. M. Hazen Co., Alburg, Vt. Embryonated eggs (White Leghorn) 8 to 12 days of age were obtained from local commercial dealers. Minced tumor tissue was placed on the dropped chorio-allantoic membrane. The window previously cut in the shell was closed and sealed with paraffin. After incubation at 35°C for 8 to 12 days the eggs were opened and the membranes were examined for growth of tumor. Histologic examination of the implanted tumor tissue and membrane was made periodically. Egg passaged tumor tissue was tested for host stability by intraocular implantation of frogs.

Results. In one experiment tumor was carried through 13 serial passages. Tumors appeared as discrete growths on the membrane and attained a size of 2-5 mm diameter. Sections of the chorio-allantoic membrane showed infiltration of the mesoderm by the tumor either in the form of cystic, papillary growths or as loosely infiltrated nests of cells. The series of passages was terminated by failure of the tumor to grow. In other attempts tumor was passed through 4 passages on 3 different occasions and through 5 and 6 passages in separate experiments. It was felt that eventual failure to grow might be associated with a tendency of the implanted tissue to become desiccated before it could become established. An attempt to avoid this difficulty was made by imbedding minced tumor fragments in chick embryo blood or adult chicken plasma clots before implantation to the egg membrane. A few drops of modified amphibian Ringer's solution (containing phosphate buffer and antibiotics) also was sometimes added onto the tissue immediately after implantation. This addition was not essential since equally good growth was obtained without it. By use of the clot technic tumor was carried through 20 serial passages. Although some tumors were as large as 10 mm in diameter the average size was 2-3 mm. Egg passaged tumor could be stored, minced in modified amphibian Ringer's solution at 4-6°C for 2 to 10 days between transfers.

Two of 4 frogs given ocular implants of tumor tissue from the 20th egg passage developed local tumor growths and had kidney tumors when examined 2 to 6 months after inoculation. Attempts to grow egg passaged tumor in the yolk sac of 5-day embryos failed. Microscopic examination of sections of the yolk sac made 13 days after implantation revealed only few, necrotic tumor cells.

Frog tumor grown at 36-37°C in roller tube, tissue cultures containing chicken plasma clot, and modified amphibian Ringer's solution grew readily in eggs after culture, *in vitro*, for 3 to 5 weeks(4).

Discussion. Heterologous transplants of amphibian tumor tissue to hens' eggs grew irregularly in serial passages. Vigorous growth of tumor in one passage might be followed by poor growth or failure to grow in the next passage or *vice versa*. It is not clear to what extent this is due to failure of the tissue to establish contact with the chorio-allantoic membrane suitable to permit nourishment of the tumor. It seems unlikely that the relatively high environmental temperature is in-

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hibitory since the tissue, *in vitro,* survived a temperature of 36-37°C for a prolonged period. The relatively slow rate of growth of the tumor tissue possibly contributes to the difficulty of implantation on the membrane. There was no evidence of alteration in the type of growth of tumor tissue during the course of 20 heterotransplants, such as was noted elsewhere during passage of frog renal carcinoma in newts(3) and during passage of a murine carcinoma in eggs(5).

Summary. The frog renal carcinoma (Lucké) has been propagated on the chorio-allantoic membrane of hens' eggs for 20 serial passages. Growth was irregular.

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Glycogen Stores in Glucagon-Treated Rats. II. Effect of Alloxan Diabetes.*

(22333)

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Chronic treatment with small doses of glucagon causes no change in liver glycogen(1), while large doses may cause a significant increase(2). Further study of this seemingly paradoxic effect of the "glycogenolytic" hormone demonstrated that, although single intraperitoneal injections of large doses of glucagon into normal rats cause an immediate decrease in liver glycogen, this is followed 24 hours later by a marked increase above normal levels(3). When the animals are sacrificed 24 hours after the last glucagon injection, as is done in chronic experiments, the short-lived glycogenolytic effects are no longer detectable because glycogen has been resynthesized. This resynthesis of glycogen may be due to 3 causes: 1) reversal of the phosphorylase reaction(3); 2) increased secretion of adrenal cortical hormones, in a manner similar to that which follows the injection of epinephrine(4); or 3) increased secretion of insulin, as a result of glucagon hyperglycemia (5). The purpose of this work was to investigate the third hypothesis by studying the effect of glucagon and epinephrine on liver glycogen, muscle glycogen and adrenal ascorbic acid in alloxan-diabetic rats.

Materials and methods. Seventy-two adult Sprague-Dawley male albino rats were used. The animals were kept on a diet of Purina checkers *ad libitum* and fasted for 24 hours before the experiments. Alloxan diabetes was produced according to the method of Sturtevant(6). Since glycogen deposits of alloxanized animals vary with the severity of the diabetes(7-9), only rats with established diabetes of more than 6 weeks' duration and with severe (4+) glycosuria and polyuria were used.‖ Glucagon (Eli Lilly and Co., lot no. 208-158B-197; 2.5 or 5.0 mg/kg),¶ epinephrine (0.15 mg/kg) and glucose (1 g/kg) were injected intraperitoneally. After 2 or 24 hours, the animals were decapitated, samples of liver and of gastrocnemius muscle and both

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‖ Alloxan diabetic rats were a gift of Dr. F. M. Sturtevant of G. D. Searle and Co.

¶ Glucagon was a gift of Dr. O. K. Behrens of the Lilly Research Laboratories.

TABLE I. Effect of Glucagon and of Epinephrine on Liver and Muscle Glycogen and on Adrenal Ascorbic Acid of Alloxan-Diabetic Rats. mg/100 g of fresh tissue. Fasting time 24 hr; glucose 1 g/kg.

No. of rats	Time after inj. (hr)	Glucagon (mg/kg)	Epinephrine (mg/kg)	Glycogen						Adrenal ascorbic acid		
				Liver			Muscle					
				Avg	SE$_m$	% change	Avg	SE$_m$	% change	Avg	SE$_m$	% change
5	2	—	—	574	± 74		387	± 42		380	± 24	
5		5	—	102	± 11	−82*	316	± 60	−19	306	± 21	−20
4	2	—	—	—	—	—	310	± 47		403	± 32	
6		5	—	—	—	—	559	± 10	+80*	290	± 15	−28*
5	24	—	—	932	± 67		413	± 24		365	± 44	
5		5	—	1045	± 94	+12	319	± 35	−23	321	± 16	−12
5	24	—	—	1348	±159		—	—	—	434	± 21	
5		5	—	1140	±101	−16	—	—	—	411	± 26	−5
5	24	—	—	639	± 84		307	± 45		326	± 18	
5		5	—	544	± 95	−16	383	± 55	+24	302	± 26	−7
4	⅓	—	—	1811	±162		212	± 47		270	± 34	
6		—	0.15	1356	± 84	−25	114	± 11	−46	211	± 22	−22
6	⅓	—	—	4180	±154		319	± 25		368	± 15	
6		—	0.15	2165	±190	−49*	205	± 28	−36*	267	± 14	−27*

* $P < 0.05$ or better.

adrenal glands were removed as rapidly as possible. Liver and muscle were placed in tared centrifuge tubes containing 30% KOH. Glycogen was determined according to the method of Good, Kramer and Somogyi(10), supplemented by the method of Nelson(11). The adrenal ascorbic acid was determined according to the method of Roe and Kuether (12). Each experiment consisted of a group of rats treated with glucagon or epinephrine and a control group treated with saline. All animals received glucose 24 hours before death. The statistical significance of the difference between mean experimental and control values was calculated according to Fisher (13).

Results. The results, presented in Table I, were as follows: 1) 2 hours after the injection of glucagon there was a marked and significant decrease in liver glycogen accompanied, in 1 experiment, by a marked and significant increase in muscle glycogen; 2) 24 hours after the injection of glucagon the liver and muscle glycogen had returned to control values; 3) in no case did liver glycogen increase above the control values; 4) no significant changes in adrenal ascorbic acid were observed after glucagon injection, except in one experiment where a significant decrease was noted; 5) twenty minutes after the injection of epinephrine there was a decrease in liver and muscle glycogen and adrenal ascorbic acid. These changes were not statistically significant; 6) 2 hours after the injection of epinephrine, the reduction in liver and muscle glycogen and adrenal ascorbic acid became more pronounced and statistically significant; 7) the average weight of the adrenal glands of alloxan diabetic animals was 38.6 mg/100 g of body weight compared to 16.3 mg/100 g in the normal controls ($P<0.01$).

Discussion. The liver and muscle glycogen content of fasted alloxan-diabetic animals is similar to that of fasted normal rats(3), indicating that well established severe alloxan diabetes does not interfere with glycogen storage. This is in accord with the observation of others(8,9,14-16). The results indicate also that alloxan diabetes does not interfere with the glycogenolytic effect of glucagon and epinephrine, but completely prevents the secondary increase in liver glycogen observed in normal animals 24 hours after the injection of glucagon and 2 hours after the injection of epinephrine. This suggests that insulin is necessary for this phenomenon. The adrenal ascorbic acid which, in the normal animal, decreases markedly following glucagon or epinephrine injections does not appear to be significantly changed in most alloxan diabetic rats. This may be due to the fact that in alloxan diabetes, a state of increased adrenal activity already exists, as suggested by the greater weight of the adrenal glands. Although hypertrophy in itself does not prove hyperfunction(17), increased adrenal cortical activity in alloxan diabetic rats has been observed also by other workers(8,18). In 1 out of 4 experiments, the injection of glucagon was followed by a significant increase in muscle glycogen, confirming previous observations in normal animals(3,19). This phenomenon appears to be contrary to the observation that glucagon inhibits the synthesis of muscle glycogen produced by insulin(20), and probably is due not to glucagon itself, but to secondary insulin or adrenal cortical secretion(3). Its occurrence in severely diabetic rats makes the first possibility unlikely. On the other hand, the decrease in adrenal ascorbic acid coincident with the increase in muscle glycogen in one experiment suggests that the phenomenon may be the result of adrenal cortical stimulation.

Summary and conclusions. 1) The effects of glucagon and of epinephrine on liver and muscle glycogen and adrenal ascorbic acid of alloxan diabetic rats were studied. 2) Twenty minutes after injection of epinephrine a decrease in liver and muscle glycogen was observed. The decrease was still greater after 2 hours. 3) Two hours after injection of glucagon there was a decrease in liver glycogen and, in one experiment, an increase in muscle glycogen. 4) Twenty-four hours after injection of glucagon, liver glycogen had returned to, but not above, the initial level. 5) The adrenal glands of alloxan diabetic rats are significantly larger than those of normal rats. The injection of glucagon or epinephrine may further stimulate adrenal cortices, as indicated by occasional decrease in their ascorbic acid content. 6) It is concluded that: a) the glycogenolytic effects of glucagon and epinephrine are unimpaired in alloxan diabetes, and b) the delayed increase in liver glycogen, observed in normal rats after injection of epinephrine or glucagon, requires the presence of an intact insulin-secreting system capable of being stimulated by epinephrine or glucagon hyperglycemia.

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Fallacy of "Crushing Death" in Frozen Bacterial Suspensions. (22334)

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It is well known that when a bacterial culture is frozen and thawed a portion of the cell population is killed. It has been generally accepted that death results from some mechanical effect produced as a result of the solidification of the suspension—either from crushing by ice crystals or from pressure resulting from ice formation(1-6). It has been demonstrated repeatedly that different species possess different susceptibilities to the lethal effect of freezing(7-9). If the lethal factor is mechanical, the differences in susceptibility to freezing must reflect differences in susceptibility to this mechanical factor. It becomes necessary, therefore, to conclude that the cells of species less susceptible to the lethal effect of freezing are physically stronger than the cells of species more susceptible. In fact, this conclusion has been presented on at least one occasion(5).

With experiments summarized in this paper we have sought to correlate the physical strength of cells with their susceptibility to freezing. In this manner, evidence for or against the ice crushing theory could be obtained. To determine the relative physical strength of different bacteria we have subjected them to violent shaking with small glass beads in the Mickle Tissue Disintegrator (MTD). Here the lethal factor is certainly mechanical, since very little death occurs in the absence of beads or abrasive, even upon prolonged shaking.

Materials and methods. Each experiment consisted of subjecting 2 different organisms to the 2 lethal conditions—successive freezings and vigorous shaking with beads. From each experiment, therefore, 4 sets of data were obtained. In every experiment *Escherichia coli* strain 69L-15 was one of the organisms employed; it was always the "reference organism" to which the behavior of a second organism was compared. The bacteria were cultivated as already outlined(9). The 2 cultures were centrifuged, the culture broth decanted, and the cells resuspended in broth of the following percentage composition: yeast extract (Balt. Biol. Lab., dehydrated), 1.0; Tween 80 (Atlas Powder Co.), 0.1; K_2HPO_4, 0.2; and $MgSO_4 \cdot 7H_2O$, 0.01. The cell concentration was adjusted (using broth) so that the viable cell count of each suspension would be approximately the same. An aliquot (7 ml) of the reference suspension (*E. coli*) was placed into a sterile vial containing 3 g of glass beads (0.057 mm average diam.).* The vial was stoppered and placed in its receptacle on one of the steel reeds of the MTD. A second aliquot (5 ml) of the suspension was placed into a sterile test tube (15 mm by 150 mm) fitted loosely with an aluminum cap. Aliquots of the "comparison suspension" were then dispensed in like manner. (Comparison suspensions were prepared using the following species: *Microbacterium flavum* strain 0J 10, *Lactobacillus fermenti* strain 69L-3, and *Bacillus pumilus* strain NRS 236.) The contents of the 2 test tubes were frozen simultaneously by placing the tubes in a –22°C alcohol bath. After 25 minutes at –22°C they were thawed simultaneously by transferring them to a +35°C water bath. A 0.1 ml sample was removed from each tube and the tubes were placed again at –22°C. In this manner, the suspensions were frozen and thawed 8 or more successive times. The 0.1 ml samples were diluted and plated in order to determine the number of cells which remained viable after a given number of successive freezings. Concurrent with the freezing-thawings, the suspensions in the MTD were being vigorously shaken. Periodically, the instrument was stopped for a time just sufficient to remove a 0.1 ml sample from each vial and to interchange the vials between the 2 receptacles. Switching the vials after each sampling was done in order to nullify the

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* Minnesota Mining and Manufacturing Co., Saint Paul, Minn.

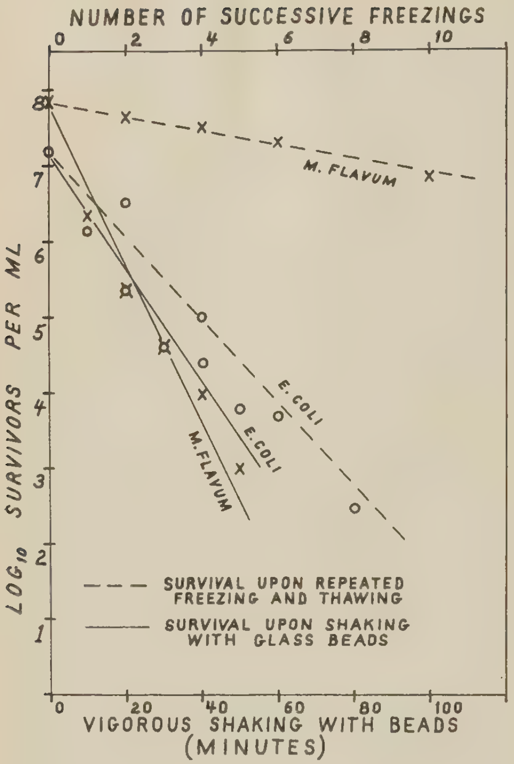

FIG. 1. Comparison of survival of *Microbacterium flavum* and *Escherichia coli* when subjected to successive freezings and when subjected to shaking with beads in the Mickle Tissue Disintegrator.

effect any small inequality in the amplitude of vibration of the 2 reeds would have upon survival of the bacteria in the respective vials. During shaking the vials were kept cool by means of a jet of CO_2. The 0.1 ml samples were diluted and plated in order to determine the number of cells which survive shaking for various periods of time. The dilution and plating technics already have been described (9,10).

Results. Fig. 1 illustrates that the susceptibility of *E. coli* to freezing is much greater than that of *M. flavum,* in spite of the fact that, as evidenced by rate of destruction in the MTD, the cells of *E. coli* appear to be physically stronger than those of *M. flavum.* In an experiment comparing *L. fermenti* with *E. coli,* little difference in the susceptibility to freezing of these 2 species was detected, although the cells of the latter species manifested a greater susceptibility to destruction in the MTD. On the other hand, in an experiment comparing *B. pumilus* with *E. coli* the cells of the former organism were less susceptible both to freezing and to shaking. There is no doubt that if sufficient pairs of species had been tested every possible combination of relative susceptibilities to the 2 lethal conditions would have been obtained. It is apparent that there is no correlation between the physical strength of cells and their susceptibility to freezing.

The susceptibility to freezing manifested by any given species is affected by both its remote and recent history. Species that are facultative toward oxygen are much less susceptible to the lethal effect of freezing when cultivated under forced aeration(9,10). In Fig. 2 it is readily apparent that cells of *E. coli* which had been aerated during growth are extremely resistant to freezing, whereas those cultivated in the customary manner show the usual susceptibility. It is important to note that the cells from the 2 cultures do

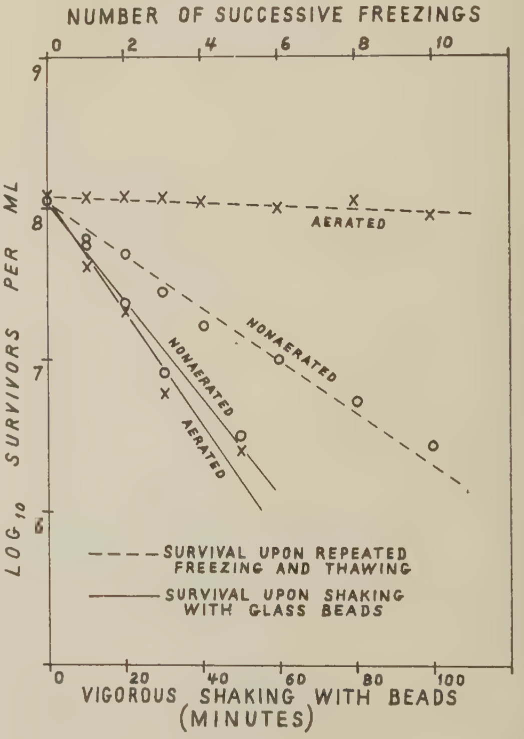

FIG. 2. Comparison of survival of aerated and nonaerated cultures of *Escherichia coli* when subjected to successive freezings and when subjected to shaking with glass beads in the Mickle Tissue Disintegrator.

not differ appreciably with respect to their physical strength. Indeed, it hardly would be expected that aeration would render cells physically stronger; rather, it might be expected that the cells would be altered in permeability and/or concentration of intracellular metabolites and enzymes. The greater resistance of aerated cells is undoubtedly due to such alterations. It is suggested that the actual cause of death upon freezing and thawing is not due to a mechanical factor but is due to some chemical or physicochemical phenomenon. The effect of solutes and initial cell concentration upon survival, as well as the very shape of the survival curves(9,10) supports this contention.

Summary. There appears to be no correlation between the physical strength of cells and their susceptibility to the lethal effect of freezing. It is therefore unlikely that the lethal factor of freezing-thawing is mechanical.

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Resistance of Specifically-Sensitized *Treponema pallidum* to Methylene Blue Stain.* (22335)

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During studies of toxoplasmosis Sabin and Feldman(1) developed a serodiagnostic test based on the observation that *Toxoplasma gondii,* which is normally stained by methylene blue, lost affinity for the dye when incubated with homologous antiserum and a thermolabile factor in normal serum. Recent experiments(2) showed that virulent *Treponema pallidum* becomes similarly resistant to methylene blue when incubated with syphilitic serum and complement. The purpose of the present studies was to explore possible applications of the reaction in the diagnosis of syphilis.

Materials and methods. The treponeme stain consisted of 0.10 g methylene blue† and 0.025 g Na_2CO_3 in distilled water to make 100 ml. It was prepared by diluting 1 ml of 2.50% Na_2CO_3 with 89 ml water, then adding rapidly 10 ml of a stock 1% solution of the dye. Both stock and dilute dye solutions were stored at 3-6°C, care being taken however to discard any showing sediment or contamination. New glass slides and coverslips, used in microscopic examination of test mixtures, were cleaned in ethanol (70%) and wiped dry with clean gauze. All other glassware was cleaned scrupulously with sulfuric-chromic acid mixture. The basic test procedure was the treponemal immobilization test(3,4). To 0.05 ml of serum in 13 x 100 mm test tube were added 0.40 ml of suspension containing 7-9 x 10^6 treponemes/ml and 0.05 ml guinea pig complement (C′); 0.05 ml of inactivated guinea pig C′ was substituted in otherwise identical serum control. The mixtures were incubated 18 hours at 35°C in an atmosphere of nitrogen (95%) and carbon dioxide (5%). The treponemes in test and control mixtures were then suspended uniformly by swirling, and samples (about 0.01 ml) transferred with capillary pipettes[‡] to opposite ends of a glass slide; delivery was effected by touching capillary tips to the slide. Two such slide preparations were made. The first served for immobilization tests, the droplets being surmounted with coverslips and given to other personnel for independent examination. An equal volume of methylene blue solution was added to the second set of droplets; individual capillaries were used for each addition and the dye and treponemes mixed with the tips. Coverslips were applied and the mixtures allowed to stand for 15 minutes at room temperature; this interval was essential for clearcut differentiation of stained and unstained treponemes. The frequency (%) of stained organisms in test and control mixtures was then determined by dark-field microscopic examination of fifty treponemes (magnification, 430 X). In this connection it should be noted that personnel familiarized with the dye and immobilization tests found the differentiation between stained and unstained organisms simpler than the decision regarding motility which was frequently complicated by sluggish or intermittently motile treponemes. The method adopted in calculating percentage of specifically dye resistant treponemes corresponded with that(4) used in determining percentage specifically immobilized; *i.e.*

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† Methylene blue chloride (dye content, 87%), National Aniline Division, Allied Chemical and Dye Corp., New York City.

$$\text{Specific dye resistance (\%)} = 100\ \frac{(\%\text{ stained in control}) - (\%\text{ stained in test})}{(\%\text{ stained in control})}$$

Comparison of dye and immobilization tests was further simplified by adopting the same convention in interpretation; *i.e.*, more than 50% specific dye resistance was considered a reaction, 21-50% a weak reaction, and less than 21% no reaction. Tests for residual C′ in each serum-treponeme C′ mixture were conducted shortly after removal of samples for dye and immobilization tests; each tube containing test mixture received 0.25 ml of a suspension containing 2×10^8 optimally-sensitized(5) erythrocytes/ml, and was incubated 30 minutes at 37°C. Fifty to 100% hemolysis was considered an acceptable result(4); less than 50% hemolysis however indicated a degree of C′ deterioration warranting repetition of test. *Syphilitic rabbit sera* were obtained from animals infected experimentally with Nichol's strain of *T. pallidum.* Samples of representative specimens were absorbed with cardiolipin antigen floccules(6); others were absorbed with virulent treponemes, the serum-treponeme-C′ mixtures being incubated anaerobically for 18 hours at 35°C and the organisms separated by centrifugation. Human sera were obtained from patients with syphilis and from individuals without clinical or anamnestic evidence of treponematosis. All were stored at –20°C. Just prior to testing, the required volume of thawed serum was heated for 30 minutes in water bath at 56°C.

Results. The results obtained in parallel methylene blue (TPMB), immobilization (TPI), and cardiolipin flocculation (CF) tests of a syphilitic rabbit serum appear in Table I A; aliquants of the same serum dilutions were used in the 3 titrations. Essentially the same degrees of reaction were observed in the parallel TPMB and TPI tests, and complement (C′) was required in both reactions (*cf* tests and C′-free controls). Attempts to absorb the serum components responsible for dye resistance or immobilization were unsuccessful, sera treated repeatedly with 6×10^7 *T. pallidum* per ml remaining unchanged in reactivity. Absorption with cardiolipin antigen floccules, however, reduced the titer of lipid flocculating (CF) antibody without affecting reactivity in the TPMB or TPI tests; the results obtained in titrations of a lipid absorbed rabbit serum are given in Table I B.

The relative sensitivity and specificity of the dye test were evaluated in a serologic study of 169 selected individuals; the TPI test was performed concurrently as a control. Table II summarizes the findings. Sixty patients presenting clinical or anamnestic evidence of syphilis showed almost identical TPMB and

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[‡] Melting Point Capillary Tubes, 1.5-2.0 x 100 mm, No. 34500, Kimble Glass Division, Owens-Illinois Glass Co., Toledo, O.

TABLE I. Treponemal Immobilization (TPI), Methylene Blue Resistance (TPMB), and Cardiolipin Flocculation (CF) Tests of a Syphilitic Rabbit Serum.

Serum dilution	TPI test: Treponemes motile, %: Test	TPI test: Treponemes motile, %: Control	TPI test: Specific immobilization, %*	TPMB test: Treponemes stained, %: Test	TPMB test: Treponemes stained, %: Control	TPMB test: Specific dye resistance, %*	CF test: Degree of reaction
A. Reactions of original serum							
1: 8	10	100	90	6	100	94	4+
1: 16	36	98	63	26	98	73	3+
1: 32	68	96	29	74	94	21	1+
1: 64	90	100	10	84	98	14	—
1:128	98	100	2	98	96	—	—
B. Reactions after absorption with cardiolipin antigen floccules							
1: 8	8	100	92	10	96	90	—†
1: 16	32	98	67	22	100	78	—
1: 32	70	94	26	74	98	25	—
1: 64	90	100	10	86	98	12	—
1:128	92	100	8	92	98	6	—

* Calculated as $100\,\frac{b-a}{b}$, where a is % of stained (or motile) treponemes in the test with complement (C′), and b is that in the C′-free serum control.

† A trace (±) reaction persisted in the test with undiluted (1:1) serum.

TPI reactions, the single exception being an individual with secondary syphilis whose serum was negative in the dye test but reacted weakly in the test for immobilizing antibody. Of special interest were 12 persons whose sera were known to yield non-specific immobilization (NSI) reactions; *i.e.*, immobilization of treponemes in C′-free controls; they were retested to determine whether factors invalidating the TPI test would interfere in the test for methylene blue resistance. All 12 sera yielded non-specific (NSMB) reactions in the dye test. In the group of non-syphilitic individuals there were 19 patients with non-treponemal infections or disorders, 7 of whom reacted serologically in tests(6,7) using cardiolipin antigens. Eighteen of the 19 proved negative in both TPMB and TPI tests. One, a patient whose serum reacted with cardiolipin antigens, was negative in the TPMB but reacted weakly in the TPI test; the diagnosis in this instance was erythema nodosum. The

TABLE II. Treponemal Immobilization (TPI) and Methylene Blue Resistance (TPMB) Tests of Sera from Syphilitic and Non-Syphilitic Persons.

Clinical diagnosis	Persons tested	TPI test: R	TPI test: wr	TPI test: —	TPI test: NSI	TPMB test: R	TPMB test: wr	TPMB test: —	TPMB test: NSMB
Syphilis									
Primary	5			2	3			2	3
Secondary	6	2	1		3	2		1	3
Early treated	5	1		4		1		4	
Congenital	3	3				3			
Neurological	24	17	1	2	4	17	1	2	4
Cardiovascular	4	2			2	2			2
Latent (probable)	13	11	2			11	2		
Diseases other than syphilis									
Reactive in STS†	7		1	6				7	
Non-reactive in STS	12			12				12	
Healthy	90			89	1			89	1

(Column heading over the TPI and TPMB tests: Persons showing given result* in the)

* Designated as reaction (R), weak reaction (wr), or no reaction (—); the symbols NSI and NSMB indicate that non-specific immobilization or dye resistance was encountered both in the test with complement (C′), and in the C′-free serum control.

† Standard flocculation and/or complement fixation tests(6,7) using cardiolipin antigens.

90 healthy persons who served as controls were young men undergoing comprehensive physical and laboratory tests as candidates for a service academy. One of this group yielded non-specific reactions (NSMB and NSI); the remainder were sero-negative.

Attempts to substitute killed *T. pallidum* in the TPMB test were generally unsuccessful. A variety of treponemacidal agents were tested. Among the chemicals found unacceptable were aqueous ethanol (5%), formalin (1%), phenol (0.1%), benzalkonium chloride (0.1%), sodium hypochlorite (0.1%), sodium ethyl mercuri thiosalicylate (0.1%), sodium citrate (2%), and a polyethylene derivative of sorbitan monooleate (Tween 80, 10%). The possibility of utilizing the treponemacidal effect of methylene blue was also explored; pre-stained organisms however proved resistant to decoloration despite prolonged incubation (18 hrs at 35°C) with syphilitic serum and complement. Heating the treponemal suspension for 10 min. at 56°C frequently immobilized the organisms without altering their capacity for specific dye resistance, but the effect was not a predictable one.

Discussion. The component of syphilitic serum conferring specific resistance to methylene blue appeared to be associated if not identical with treponemal immobilizing antibody. This conclusion was suggested by the close agreement observed in quantitative dye and immobilization tests and by the common requirement for complement (Table I). It was supported by almost identical distribution of reactions in qualitative TPMB and TPI tests of patients in the various stages of syphilis (Table II). The relationship of the responsible serum component to *T. pallidum* was not demonstrable by specific absorption, an experience which has an interesting parallel in the recent failure to neutralize treponemal immobilizing antibody with soluble antigen(8). Whatever its nature, however, the essential factor in syphilitic serum was distinct from the antibody concerned in reactions with cardiolipin antigens (Table I). As such it provides an additional basis for differentiating the patient with latent syphilis from the uninfected individual whose serum reacts with lipid antigens; evidence of the usefulness of the TPMB test in this respect was obtained in the present study (Table II). Further evaluation of the test is essential, however, and continuing studies should emphasize particularly the development of a killed treponemal suspension exhibiting the specific dye resistance characteristic of live *T. pallidum.* Being based on specific alterations in the treponemal staining reaction, the TPMB test has certain advantages over one(3,4) relying on changes in motility, and the development of a stable, killed antigen would mark an important advance in standardization.

Summary. Virulent *T. pallida,* which are normally stained by methylene blue, become resistant to the dye following incubation with syphilitic serum and complement. The responsible factor in syphilitic serum is associated if not identical with treponemal immobilizing antibody. The reaction provides the basis for a specific diagnostic test for syphilis.

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Coenzyme A Activity in Tissues of Normal and Scorbutic Guinea Pigs.

(22336)

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By using labelled acetate Bloch *et al.*(1-4) showed that cholesterol is formed in the body from acetate. Adrenal cholesterol diminished in scurvy(5). Scorbutic guinea pigs excreted a lesser amount of injected p-aminobenzoic acid (PABA) in the acetylated form(6). Diminished adrenal cholesterol in scorbutic animals seemed possibly due to diminished acetylation in the body. But it was also observed(6) that while cholesterol content of adrenals, spleen and lungs diminished, that of testes and small intestine increased and there was no change in the cholesterol content of liver and kidney(6). It was, therefore, not clear if the total synthesis of cholesterol by a scorbutic guinea pig was altered. Becker *et al.*(7) reported that liver and adrenals of scorbutic guinea pigs fed labelled acetate contained cholesterol with higher specific activity, which indicated that adrenal glands of scorbutic guinea pigs synthesized more cholesterol from administered acetate. It was, therefore, difficult to explain why scorbutic guinea pigs showed diminished acetylation reaction. Decreased acetylation of PABA might be due to diminished coenzyme A (Co A) activity of tissues. The present investigation was undertaken to study Co A activity of tissues of scorbutic and paired fed normal guinea pigs. Cholesterol content of the whole body of the animals was also determined.

Methods. Production of scurvy. Selection of guinea pigs and feeding a scorbutic diet, with or without the supplement of ascorbic acid by the paired feeding technic, was the same as described previously(5). *Studies with urine.* Acetylation studies were carried out as described previously(6). Results are given in Table I. *Adenosinetriphosphate* (ATP) was prepared as dibarium salt by the method of Dounce *et al.*(8) from rabbit muscle. Before use, the barium salt was converted into potassium-ATP. *Preparation and aging of apoenzyme from pigeon liver.* Apoenzyme was prepared from pigeon liver and aged by the method of Kaplan and Lipmann (9).

TABLE I. Percentage of Acetylated PABA Excreted in Urine by Guinea Pigs. Average of 3 days excretion.

Normal 25.0 ± 2.4* (5)	Scorbutic 17.4 ± 2.5 (5)	t: 2.2

* Stand. error.
Figures in parentheses indicate No. of animals.

Results. Studies with tissues. Scorbutic animals were sacrificed on the 25th day of experiment along with their paired fed controls and Co A activity of liver, kidney and brain was determined by the method of Kaplan and Lipmann(9). Dry weights of the tissues were determined and Co A activity was expressed on dry weight basis. No Co A preparation of known potency was available. Crude preparation of Co A was obtained by the method of Kaplan and Lipmann(9) from rabbit liver and was used as the reference Co A. Activity-concentration curve was prepared with this reference Co A for each batch of extract of apoenzyme over a wide range including the saturation point. Half of the maximum activity was taken as unit. From this standard curve the Co A contents of the test samples were obtained directly. Results are given in Table II.

Estimation of total body cholesterol. Animals were killed, gastrointestinal tracts washed, whole body minced, weighed, dried

TABLE II. Coenzyme A Contents of Tissues of Normal and Scorbutic Guinea Pigs. Lipmann units of Co A/g dry tissue.

	Liver	Kidney	Brain
Normal (9)	959 ± 58	420 ± 35	294 ± 27
Scorbutic (9)	1007 ± 54	500 ± 40	301 ± 21

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* One of us (S.L.) received Kalyan Kumar Mukherjee Research Scholarship from Calcutta University, India.

TABLE III. Total Body Cholesterol of Normal and Scorbutic Guinea Pigs. mg/100 g fresh tissue.

Normal	219 ± 5.7 (8)	Scorbutic	269 ± 9.8 (8)	t: 4.4

at 85°C for 16 hours, powdered and extracted with acetone in a Soxhlet apparatus for 24 hours. Cholesterol was estimated in aliquots of extracts by the method of Sobel and Mayer (11). Results are given in Table III.

Discussion. Scorbutic guinea pigs showed decreased acetylation of administered PABA. Co A activity of tissues of scorbutic guinea pigs did not change. As the paired feeding technic was employed the consumption of pantothenic acid was similar in both the groups of animals. The decreased excretion of injected PABA in acetylated form by scorbutic guinea pigs indicated that ascorbic acid was in some way concerned in acetylation reactions in the body. A significant increase in the whole body cholesterol of scorbutic guinea pigs was observed. This might be due to either increased synthesis or decreased utilization of cholesterol in the body of a scorbutic guinea pig. The high rate of incorporation of acetate into cholesterol by the livers of alloxan-diabetic rats is reduced by insulin(12). Recent observations indicate that in insulin insufficiency and also in vit. C deficiency the oxidation of acetate in the tricarboxylic acid cycle is hampered(13-15). Vit. C deficiency in turn decreases the insulin content of pancreas(16). Thus acetate pool which is not burned through tricarboxylic acid cycle might be utilized in the increased formation of cholesterol. The increased cholesterogenesis in the scorbutic guinea pigs, therefore, might be due to diminished insulin synthesis.

Summary. Acute scurvy was produced in guinea pigs by paired feeding technic. Urinary excretion of injected PABA in the acetylated form was estimated in scorbutic and paired fed normal guinea pigs. Scorbutic animals excreted decreased amount of acetylated PABA. Co A activity of liver, kidney and brain of scorbutic and paired fed control guinea pigs was determined. Co A activity which was present in the tissues of normal guinea pigs did not change in scurvy. Total body cholesterol of scorbutic guinea pigs increased significantly. The implications of the results obtained have been discussed.

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In vitro Conversion of Testosterone to 17β-Hydroxyandrostan-3-one.* (22337)

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In schemes for the metabolism of testosterone, a theoretical intermediate which has been postulated but up to now not demonstrated is 17β-hydroxyandrostan-3-one(1). In this paper evidence is presented for the formation of this steroid when testosterone was incubated with a homogenate of liver from female rats.

Method. Two incubations were carried out. In both, female Sprague-Dawley rats weighing 200-250 g were decapitated, and the livers removed and homogenized immediately in a Potter homogenizer. The homogenizing medium was that described by Samuels and West (2). The incubations were carried out in a Dubnoff shaking incubator, in air, at 38°C. 1.5 ml of homogenate were added to 20 ml beakers, containing 1 mg testosterone in 0.06 ml propylene glycol, and 3 mg diphosphopyridine nucleotide (DPN) were added just before incubation.

Incubation No. 1. One gram of liver was homogenized per one ml of medium and the gauze-filtered homogenate was diluted with an equal amount of homogenizing fluid. There was approximately one-half gram of tissue per incubation flask. Twenty beakers were incubated for 90 minutes. After incubation, the reaction was stopped by addition of 0.15 ml 3 M acetate buffer, pH 5. The incubation mixtures were pooled and extracted 3 times with ethyl acetate. The ethyl acetate extract was dried over sodium sulfate, filtered, and the solvent removed *in vacuo*. Without any further purification, this extract was chromatographed on a 14 cm paper strip, in the ligroin-propylene glycol system(3) for 27 hours. A zone giving a blue Zimmermann color, having a mobility slower than that of androsterone, was eluted. This eluted material was taken up in chloroform, washed with a small volume of water, and dried over sodium sulfate. After removal of the chloroform, *in vacuo*, it was analyzed by infrared spectroscopy† and found to contain 17β-hydroxyandrostan-3-one as the major component. The crude material weighing 8 mg was rechromatographed in ligroin-propylene glycol for 24 hours, with crystalline 17β-hydroxyandrostan-3-one as standard on an adjacent strip. The mobilities of the isolated material and standard were the same. The eluted material was crystallized from acetone and methanol and melted at 175-178°C. When mixed with an authentic sample melting at 178°, 182-3° the mixture melted at 175-8°C. The melting point was depressed to 148°-167°C when mixed with androsterone melting at 181-4°C. The infrared spectrum of the isolated crystalline material was identical to that of the authentic sample.

Incubation No. 2. 13 g of liver were homogenized to a total volume of 35 ml. This homogenate contained about 0.56 g of tissue per 1.5 ml. Twenty beakers were incubated. After 2 hours incubation, 4 groups of 5 incubation mixtures were pooled, and 150 ml of acetone were added to each pool. After shaking for an hour, the acetone extracts were filtered and evaporated to dryness, *in vacuo*. The acetone extracts were dried overnight in a vacuum desiccator, over calcium chloride. The extracts were chromatographed for 20 hours in ligroin-propylene glycol. The zones whose mobilities were slower than that of androsterone were pooled and rechromatographed for 20 hours and the major Zimmermann-reacting (blue) zone was eluted for infrared analysis. The major component was identified as 17β-hydroxyandro-

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† Infrared analyses were carried out by Mr. Paul Skogstrom under the direction of Dr. Harris Rosenkrantz.

stan-3-one.

Summary and conclusion. Incubation of testosterone with an homogenate of liver from female rats led to formation of 17β-hydroxyandrostan-3-one. This steroid has been postulated as a possible intermediate in the metabolism of testosterone but up to now has been neither isolated from natural sources nor detected in metabolism experiments *in vivo* or *in vitro*.

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Clinical, Bacteriological, and Serological Observations of two Human Volunteers Following Ingestion of *Escherichia coli* O127:B8. (22338)

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An epidemic of infantile diarrhea due to *Escherichia coli* O127:B8 reported by Stulberg and co-workers(1) has been further studied serologically by Stulberg, Zuelzer and Page(2). In an effort to produce specific antibodies in humans to be used as positive control sera for the serological portion of their studies, 2 adult volunteers ingested O127:B8 organisms following the methods employed by Ferguson and June(3) and June, Ferguson and Worfel(4) in their classical feeding experiments using *E. coli* O111:B4 and O55:B5. Since volunteer studies using O127:B8 have not been published, and because clinical, bacteriological and serological results of seeming interest were obtained, this experience is being reported.

Methods. Two adults, white males, age 28 and 32, participated in this study. Each man was in general good health and had been free of diarrhea of any nature for at least one year prior to this experiment. *Inocula*: *E. coli* O127:B8 cultures obtained from 3 infants during their diarrheal disease(1) were inoculated separately on brain heart infusion agar slants. After 18 hours incubation at 37°C, the growth from each slant was harvested in saline. The 3 suspensions were pooled and washed once in saline. The pooled suspension was diluted in saline and colony counts estimated the inocula for the two volunteers to contain 4,000,000 and 8,000,000 organisms respectively. In each instance the inoculum was contained in 1 ml. Each inoculum was added to one-half pint of pasteurized milk which was kept on ice until ingested, approximately one hour later. A challenge inoculum of 8,000,000 organisms prepared in a similar manner was ingested by volunteer A 32 days following the initial ingestion. One pre-ingestion blood sample and 3 pre-ingestion rectal swabs were obtained from each volunteer. Following ingestion, rectal swabs and venous blood samples were collected daily for about 2 weeks and later at approximately 3 day intervals. A detailed record of symptoms was kept by each volunteer. *Bacteriological and serological examinations*: Rectal swabs were inoculated immediately on blood agar and MacConkey's bile salts agar plates. After 20 to 24 hours, colonies appearing on the blood plates were tested for agglutinability with OB rabbit antiserum prepared with *E. coli* O127:B8, strain 9782 obtained from Dr. William Ferguson. Standard bacterial agglutination tests using OB and O antigens prepared with *E. coli* O127:B8 culture No. 2220 were performed according to the method described by Kauffmann(5). Saline suspensions of the organisms grown for 18 hours on brain heart infusion agar were used as antigens. Hemagglutination tests were performed by a technic developed by Stulberg, *et al.*(2), based on the methods of Neter, *et al.*(6), and Wheeler and Taylor(7).

Results. Symptoms: Both volunteers experienced a sharply defined illness beginning with an explosive liquid stool. Liquid diarrhea, abdominal cramps, nausea and mild to moderate malaise were the predominating symptoms. In volunteer A the incubation period was 17 hours, the illness lasted 32 hours and with 4 diarrheal stools and an afebrile course was classified as mild. In volunteer B the incubation period was 7 hours, the duration 46 hours and with 10 diarrheal stools and a per oral temperature elevation of 100°F, the illness was classified as of moderate severity. No symptoms were recorded following the challenge ingestion by volunteer A.

The presence of *E. coli* O127:B8 in fecal cultures was determined by slide agglutination tests with 5 to 15 colonies from each blood plate. Organisms of this serotype were recovered from both volunteers 24 hours after ingestion and for a period of 5 days in one volunteer and 12 days in the other. In volunteer A, *E. coli* O127:B8 was found in the stools in increasing amounts and on the sixth day after ingestion, was the only organism present. The stool flora then gradually reverted to its pre-ingestion status. From the 13th through the 26th day 5 negative cultures were obtained. On the 32nd day a pre-challenge ingestion culture produced scanty growth of O127:B8. One day following the challenge ingestion moderate growth was present. Additional post-challenge cultures were not obtained.

Serological examination: Results of the standard agglutination and hemagglutination tests are shown in Table I. Serum hemagglutinins were detected as early as 3 days after ingestion and bacterial agglutinins with boiled cultures within 5 days. Titers with both tests continued to rise until the eighth or ninth day and remained near the peak level for the remainder of the initial experiment. Bacterial agglutinations with living cultures were, in every instance, negative at a serum dilution of 1:10. In volunteer A on the 32nd post-ingestion day bacterial agglutination was negative at a serum dilution of 1:10 while the hemagglutinin titer had dropped to 1:40. Three days following the challenge ingestion bacterial agglutinin and hemagglutinin titers were 1:40 and 1:160 respectively.

TABLE I. Results of Serological Examinations.

	Agglutination titers* at different intervals					
	Volunteer A			Volunteer B		
Days following ingestion	Bacterial O	Bacterial OB	Hemagglutination	Bacterial O	Bacterial OB	Hemagglutination
0	0†	0	20	0	0	10
1	0	0	20	0	0	10
2	0	0	40	0	0	20
3	0	0	80	0	0	40
4	0	0	80	0	0	40
5	10	0	80	10	0	40
6	20	0	320	20	0	80
7	160	0	320	40	0	80
8	80	0	320	40	0	160
9	160	0	640	40	0	160
12	80	0	320	40	0	80
15	80	0	320	20	0	80
18	80	0	320	20	0	80
32	0	0	40	nt		nt
3 (post-challenge)	40	0	160	nt		nt

* Titers are shown as reciprocal of highest dilution in which definite agglutination was observed.

† Zero indicates negative result at dilution of 1:10.

nt = not tested.

Discussion. Our results are interpreted as evidence of a definite bacterial, clinical and serological response by 2 adult human volunteers to the ingestion of *E. coli* O127:B8 organisms. The clinical responses, including the incubation periods and the duration of symptoms, are similar to those observed by Ferguson with serotypes O111:B4 and O55:B5. It is interesting to note, however, that volunteer B suffered an illness of moderate severity after the ingestion of only 4,000,000 organisms. In no instance did Ferguson record an illness of more than slight severity with dosages of less than 530,500,000 organisms with O111:B4 or 143,000,000 organisms with O55:B5. The question of possibly greater pathogenicity for adults of serotype O127:B8 in comparison with O111:B4 and O55:B5 is raised by these observations.

Serological tests showed comparable rises in antibody titer for the 2 volunteers, the hemagglutinin titers being consistently higher than the bacterial agglutinin titers. This is in agreement with the results obtained by Neter(8) in a comparison of the two methods, using sera obtained from volunteers in the feeding studies of Ferguson. The antibody response detected by bacterial agglutination when using boiled cultures as antigen could not be demonstrated when living cultures were used. This is in accord with Ferguson's results.

In both volunteers *E. coli* O127:B8 appeared in the stool 24 hours after ingestion of the cultures and increased in numbers during the following few days. The persistence of the organisms in substantial numbers for several days after the complete subsidence of symptoms is interesting and is in accord with the findings of Ferguson and co-workers with the O111:B4 and O55:B5 serotypes.

The results of the challenge feeding suggest that protection from clinical symptoms may have been afforded by the initial ingestion. It is interesting that the pre-challenge culture was positive although 5 previous cultures had been negative. Properly designed human experiments seem indicated to study a possible post-ingestion immune response and to delineate the duration and consistency of fecal elimination of the organism during convalescence.

Summary. 1. Two healthy, adult white, male volunteers ingested, respectively, 4 and 8 million organisms of *E. coli* serotype O127:B8. 2. Symptoms of mild to moderate gastroenteritis appeared in both subjects 7 to 17 hours after ingestion of the organisms and persisted for 32 to 46 hours. 3. By daily rectal swab cultures, *E. coli* O127:B8 organisms were recovered within 24 hours after ingestion and were found during 5 days in one and 12 days in the second volunteer. 4. Hemagglutinins were detected 3 days and bacterial agglutinins 5 days following ingestion. Antibody titers in both subjects rose rapidly, reaching peaks by the eighth to ninth day. Hemagglutination titers were consistently higher than bacterial agglutination titers. 5. Challenge ingestion of 8,000,000 organisms by one volunteer was followed by bacterial and serological response without appearance of clinical symptoms.

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Relation of Glucagon to A Cells of the Pancreas.*† (22339)

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In spite of the amount of work done on origin of glucagon, there is no agreement as to the site of its production(1-5). Some investigators found a significant reduction of glucagon in the pancreas of cobalt-treated guinea pigs and dogs(8,9); others found no diminution in glucagon content of cobalt-treated dogs(6,7); while others stated that the glucagon content of cobalt-treated rabbits was twice that of normal(4). However, these workers(4) found no glucagon in the pancreas of synthalin A treated rabbits. Some but not all controversies on origin of glucagon could be explained by differences in toxic effect on the A cells of different animals(10,11). It has recently been demonstrated by one of us that the uncinate process of the dog pancreas is normally devoid of A cells(12) and that no significant hyperglycemic effect can be obtained with extracts from such an area while there is a marked hyperglycemic effect with extracts from the A cell containing portion of the pancreas(13). Because of the specific morphological differences in the dog pancreas, it was thought that other differences rather than the presence or absence of A cells might explain the regional differences in the glucagon content of the dog pancreas. This hypothesis had to be seriously entertained in view of the conflicting reports on the relation of glucagon to the A cell(1-5). To clarify this relationship, an attempt was made to destroy the A cells of the dog pancreas with cobalt but unfortunately we were unable to do so, although they were easily damaged in the guinea pig(11). Experiments were carried out then with the object of producing complete disappearance of the A cells of the guinea pig with the hope that a pancreas with these changes would be analogous with respect to its morphology and glucagon content to that of the uncinate process of the dog.

Material and methods. A total of 64 guinea pigs of both sexes were divided as follows: *Group 1:* Five guinea pigs weighing between 600 and 1200 g were used for the study of possible regional differences in the distribution of islet cell types in the guinea pig. The entire pancreas of these animals was embedded and sectioned and in none of the representative sections could we find evidence of any regional differences. *Group 2:* The pancreases of 4 untreated guinea pigs weighing between 900 and 1200 g were pooled and used for glucagon extraction. A small portion of each gland was first taken for microscopic examination. *Group 3:* Four guinea pigs weighing between 400 and 700 g and 6 guinea pigs weighing between 1100 and 1400 g received daily subcutaneous injections of 20 mg of cobalt chloride (B.D.H. Analar) per kg of body weight as a 1% solution for as long as 7 days. The animals of the younger group were all killed at the seventh day. Of the older group, 5 died between the third and fourth days; the survivor was killed on the fifth day. The entire pancreas of all these animals was embedded and studied as in Group 1. The older animals showed the most extensive A cell lesions, but were also more susceptible to the lethal effect of cobalt. The extent of damage to the A cells was variable from one animal to another but of similar degree throughout the pancreas of any one guinea pig. On this basis in subsequent experiments the extent of A cell damage of the portion of pancreas used for glucagon extraction was assumed to be identical to the small one used for histological examination. *Group 4:* Because most animals lost about 10% of their weight during the cobalt treatment 9 guinea pigs were partially starved to make them lose 10% of their weight within 5 days so as to exclude weight

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loss as the cause of the A cell lesion in the cobalt treated guinea pigs. None of these starved animals showed A cell damage. *Group 5:* Thirty-six guinea pigs weighing between 900 and 1200 g were treated with cobalt chloride as in Group 3; fourteen died and were discarded, the remaining 22 were killed between the fifth and seventh day after the beginning of the treatment. A small portion of the pancreas was obtained from each of these animals for microscopic study, the remainder of the organ was used for glucagon extraction. Histologically, the extent of the A cell lesion was graded for each pancreas from 1+ to 4+. Eight animals had 1+; 5 had 2+; one had 3+; and 8 had 4+ lesions of their A cells. Extracts from animals having the same degree of damage were pooled in groups up to 4. All animals had free access to water and Purina rabbit chow supplemented with fresh lettuce and carrots. All guinea pigs were killed at 6 hours after the last injection of cobalt by exsanguination under ether anesthesia. Glucagon was extracted by the method of Best, *et al.*(14), each pancreas was extracted individually and then pooled as described above. The pooled extracts were dried by evaporation in the cold. Every pooled extract was tested in triplicate following the procedure suggested by Staub and Behrens(15). The presence of glucagon was demonstrated by its hyperglycemic effect on fasted, anesthetized cats. The dry extract in an amount equivalent to 2 g of fresh pancreas was dissolved in 2 ml of normal saline (pH 2.5) immediately before injection. The solution was injected intravenously and blood samples were taken prior to and 5, 10, 15, 20 and 25 minutes after the injection. Individual blood sugar determinations were done in duplicate with a modification of the Folin-Wu method. The extract from the animal with a 3+ lesion was tested only once because of insufficient material for more determinations. Tissues for histological examination were fixed in Zenker-Formol, and were processed and stained following the methods previously described(16). Paraffin sections were cut 2.5 mu thick and stained with Gomori's chrome alum hematoxylin stain, the aldehyde fuchsin stain of Gomori(17) and a recent modification of Masson's trichrome(11).

Results. Morphology. The A cell lesion of the cobalt-treated guinea pig was qualitatively identical in all animals. Cobalt produces a progressive and complete degranulation of the A cell, while its cytoplasm and nucleus remain unaffected. With further treatment, the degranulated A cell undergoes vacuolization of its cytoplasm (Fig. 1,2). It was not possible, however, to see any necrotic cells in the pancreases of cobalt-treated guinea pigs. Our grading of the A cell lesions was based on the proportion of A cells showing complete degranulation or more advanced changes. We considered a 1+ lesion to be present in all those cases in which some cells were observed. When about half of the A cells were so damaged, it was graded as a 2+ lesion. When only a few granulated A cells could be found, the lesion was classified 4+. An attempt was made to interpolate a 3+ lesion, but this could be done on only one occasion.

Glucagon content of the pancreas of untreated guinea pigs: The extracts from the pancreases of these animals invariably showed when tested a marked hyperglycemic effect on the fasted anesthetized cats. Their blood sugar rose from 50 to 153 mg% above the initial level within 15 minutes after the injection of the extracts as indicated in Fig. 3.

Glucagon content of cobalt-treated guinea pigs: When tested, pancreatic extracts from animals having 1+ and 2+ lesions showed a response similar to that of the untreated animals. As noted in Fig. 3, the extracts from a 2+ lesion gave a rise of blood sugar varying from 73 to 104 mg %. In contrast with these results the extract from animals having a 4+ lesion showed on only one occasion a rise of 4 mg % above the initial level. This was followed by a decrease in the blood sugar from 40 to 62 mg % within 25 minutes. The pancreatic extract from the animal having a 3+ lesion showed a rise of blood sugar of 16 mg % above the initial level.

Discussion. An important finding in the present work is the lack of a hyperglycemic effect of the pancreatic extract from animals having 4+ lesions, particularly since the hy-

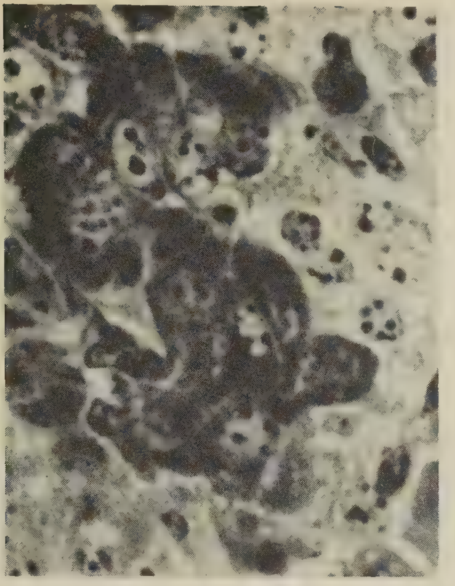

FIG. 1. Pancreatic islet from normal guinea pig showing well granulated A cells in black. Masson's trichrome × 900.

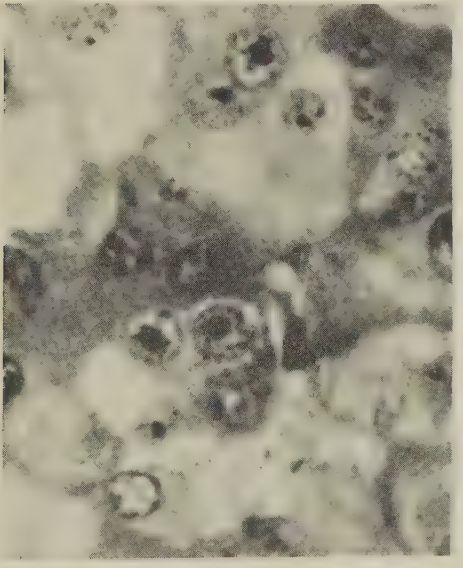

FIG. 2. Pancreatic islet from a cobalt-treated guinea pig. A cells are severely degranulated or vacuolated. They appear clear in the picture. Masson's trichrome × 900.

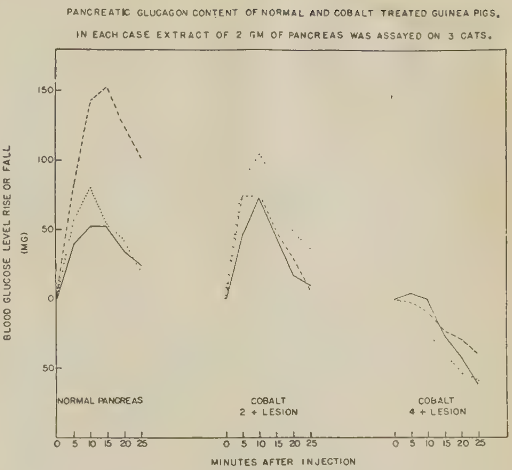

FIG. 3. Typical blood sugar curves in fasting and anesthetized cats after intravenous injection of pancreatic extracts from normal and cobalt-treated guinea pigs.

perglycemic effect of the extract from animals having a 1+ and 2+ lesion is indistinguishable from that of the untreated ones. Our results suggest that the pancreatic extracts from cobalt-treated guinea pigs fail to elicit a hyperglycemic effect, as is the case with extracts from the A cell-free portion of the dog pancreas when the number of granulated A cells present is reduced to a certain minimum which apparently is achieved only in our 4+ lesion. Because 4+ lesions occurred in only 40% of cobalt-treated animals, it is possible that the 60% reduction of glucagon content obtained by Vuylsteke *et al.*(8) in their pooled extracts from pancreases of cobalt-treated guinea pigs was due to the fact that these authors did not grade the extent of their lesion. From our experience with cobalt in various species(11) from the work with the uncinate process of the dog(12,13) and since none of the substances toxic for the A cell have resulted in pancreas completely devoid of them(1,3,4), it seems that the conflicting results on the relation of glucagon to the A cell may be due to the great hyperglycemic activity of minute amounts of glucagon remaining in the pancreas when a few granulated A cells are present. We are, however, unable to explain on the basis of our results the work of Fodden and Read(4) who found that extracts from cobalt-treated rabbits had double the normal amount of glucagon while that of synthalin-treated ones had none despite the fact that A cells were severely damaged in both groups. It is unfortunate that these investigators did not make a detailed comparison of the A cell lesion as it occurred in the rabbits treated with these two substances. The results of our work are interpreted as strongly suggestive that glucagon originates from the A cells. Quantitative determination of the glucagon content of pancreas treated with drugs toxic for the A cell together with detailed morphological study of the type of A cell lesion, will aid in the elucidation of this problem.

Summary. Pancreatic extracts of cobalt-treated guinea pigs with a 4+ lesion fail to produce a significant hyperglycemia when assayed in cats; however, if well-granulated A cells remained in amounts roughly estimated above 25% of the normal, the extracts from these pancreases invariably elicited a hyperglycemia indistinguishable from that of the normal. These results are interpreted as strongly suggestive that glucagon originates from the A cells.

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Effect of Splenectomy on Antibody Formation in Cortisone Treated Rats. (22340)

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The hemolysin formation in rats after a single intraperitoneal injection of sheep erythrocytes was the object of a previous study (1). It was suggested that at a large dose of the antigen (about 2 ml of a 10% suspension) a considerable part of the sheep cells enters the blood stream and is taken up by the spleen. The spleen may then be responsible for a large fraction of the circulating antibodies measured. However, at an antigen dose 30-40 times smaller, splenectomy preceding(2,3) or subsequent to (unpublished experiment) the intraperitoneal antigen injection did not appreciably alter the hemolysin response. In another series of experiments some factors which condition the antibody-reducing effect of cortisone were studied(4). It was shown that the hemolysin response after a large intraperitoneal dose of sheep cells was less affected by cortisone than that after a small one. One possible explanation involved the assumptions: 1) that the spleen produces a dominating part of the hemolysins with a large antigen dose but not with a small one, and 2) that splenic antibody production is more resistant to cortisone than is extrasplenic antibody formation. The effect of cortisone should then be less with a large than with a low antigen dose.

The following experiment was an attempt to verify the two assumptions.

Material and methods. Animals. White

TABLE I. Effect of 4 mg Dose of Cortisone on Hemolysin Response in Sham Operated and Splenectomized Rats. Animals were immunized by one intraperitoneal injection of sheep erythrocytes on day 0.

Group	Operation	Treatment	Initial avg wt, g	Hemolysin titer, mean ± stand. error of mean†		
				Day +4	Day +5	Day +8
I	Sham	C* day −8 to +1	208	3.26 ± .52 (9)	6.81 ± .38 (9)	6.77 ± .55 (9)
II	”	No	204	5.19 ± .45 (8)	7.50 ± .29 (8)	6.41 ± .36 (8)
III	Splenectomy	C day −8 to +1	200	.00 (10)	.57 ± .37 (10)	1.40 ± .62 (9)
IV	”	C day −2 to +1	196	.07 ± .03 (9)	2.77 ± .47 (9)	3.94 ± .49 (9)
V	”	No	214	1.87 ± .49 (7)	5.57 ± .43 (7)	5.37 ± .48 (6)

* C = Cortisone. † Figures within parentheses are No. of animals.

male rats weighing 170-200 g were allotted at random to the experimental groups(1). Blood samples of 1 ml were taken from tails. *Splenectomy and sham operation.* All operations were performed under evipal sodium anesthesia on the 1st day after the antigen injection. After midline abdominal incision the spleen was exteriorized. In the sham operation, the spleen was replaced in the abdomen. When splenectomy was performed the spleen was removed after ligation of the pedicle. *Hormone.* Cortisone acetate* was given as daily intramuscular injections in a dose of 4 mg per 100 g body weight (designated "the 4 mg dose"). *Antigen.* Fresh sheep erythrocytes were washed 3 times with saline and diluted with saline to a 10% suspension. A small quantity of India ink (dilution in antigen 1:2000) was added. The rats were immunized by a single intraperitoneal injection of 1 ml of the antigen suspension per 100 grams initial body weight (Table I). At the end of the experiment all animals were autopsied to determine whether antigen had been deposited in the peritoneal cavity (traces of carbon). The day of antigen injection is called day 0, days before and days after are conventionally indicated by minus and plus respectively. *Titration of hemolysin.* Inactivated sera were titrated in 2-fold serial dilutions. Hemolysins were determined by a standard procedure(1) using 4 units of complement, incubation at 37°C for 30 minutes, and subsequent centrifugation. The degree of hemolysis was estimated by the aid of a hemolytic scale. The 50% end point was calculated by interpolation. The titers are expressed as the negative ^{2}logarithms of the serum dilution, taking dilution 1:6.25 as a provisional zero point (1:12.5 = 1; 1:25 = 2, etc.). All sera from one bleeding were titrated simultaneously.

Experimental. A scheme of the cortisone treatments and the operations is given in Table I. Two groups of 12 rats were given the 4 mg dose of cortisone for 10 days beginning 8 days before the antigen injection (I and III). One group of 9 rats received the same dose of cortisone for 4 days beginning 2 days before the antigen injection (IV). Another 2 groups of 9 rats had no treatment (II and V). One day after antigen injection, splenectomy was performed on the following groups: Group III, treated with cortisone for 10 days; Group IV, treated for 4 days, and Group V, non-treated. The remaining 2 groups (I and II) were sham operated. Five animals died during or soon after the operation. Another 5 animals died later, probably due to infections. In 3 of these the intraperitoneal deposition of the antigen could not be verified. The data of these rats have been excluded. The animals were bled on the 4th, 5th and 8th day after the antigen injection.

Results. Splenectomy produced a significant reduction of the hemolysin level on day +4 (Fig. 1 and Table I). This effect appeared to be less pronounced on day +5 and was no longer significant on day +8.

In sham operated animals the 10-day cortisone treatment produced a probably significant ($P<0.02$) titer reduction on day +4 (Table II). At the later bleedings this effect was no longer manifest. In splenectomized rats the same treatment had a significant effect on all bleeding days. On day +5 and +8 the 10-day cortisone treatment seemed to be much more effective in splenectomized

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* Cortone, Merck and Co., furnished through kindness of Erik Lindblom and Co., Stockholm.

TABLE II. Mean Titer Difference between Non-Treated Groups (II and V) and the Respective Similarly Operated Cortisone-Treated Groups (See Table I).

Operation	Cortisone, No. of days	Titer reduction, mean ± S.E. of mean Day +4	Day +5	Day +8
Sham	10	1.93 ± .69*	.69 ± .49	−.36 ± .67
Splenectomy	10	≥1.87 †§	5.00 ± .57‡	3.97 ± .86‡
"	4	1.80 *§	2.80 ± .66‡	1.43 ± .72

* Significance level 5%. † Significance level 1%. ‡ Significance level .1%.

§ This significance was obtained by comparison of intra-group distributions of values above and below the general mean of the two groups, see Mainland(7).

animals than in those sham operated. To test the significance of this difference, an analysis of variance(5) was used with two bases of classification: splenectomy and cortisone treatment (10 days or none). The interaction term proved the difference significant at the 0.001 level.

In splenectomized animals cortisone treatment for 4 days produced a significant reduction of the hemolysin level on day +5 but not on day +8 (Table II).

Discussion. Splenectomy produced a significant reduction of the hemolysin level only on the 2 first bleeding days (Fig. 1). Thus the splenectomized animals seemed to have a slower rise of antibody but may actually have formed as much antibody as the controls. Taliaferro and Taliaferro(6) have reported similar observations in rabbit experiments where the antigen was given intravenously. These authors suggest "that the spleen in intact animals usually forms a large part of the antibody which accumulates to give the initial peak serum titer, and then abruptly ceases to form antibody." Such an early transient role of splenic hemolysin formation may also explain the present findings.

Administration of the 4 mg dose of cortisone for 10 days, begun 8 days before the antigen injection, was significantly more effective in splenectomized than in sham operated animals on day +5 and +8. In fact cortisone treatment for 4 days, started 2 days before the antigen injection, was enough to produce a significant reduction of the antibody level on day +5. In non-splenectomized animals this short period of hormone administration has failed to significantly affect the hemolysin response to this antigen dose (about 2 ml of a 10% suspension) but has repeatedly suppressed the antibody level on day +5 at a 30 times smaller sheep cell dose (4).

The present results indicate that splenectomy has lowered the resistance to cortisone of the hemolysin formation after a large intraperitoneal antigen dose. The mechanism of this effect is not clear. The result of splenectomy in animals not treated with cortisone (c.p. day +8, Fig. 1) does not lend support to the hypothesis that most of the hemolysins, produced after a large antigen dose, emanate from the spleen. Consequently the present data do not indicate whether or not the splenic antibody formation is more resistant to cortisone than the extra-splenic. The experiment has not clarified the cause of the

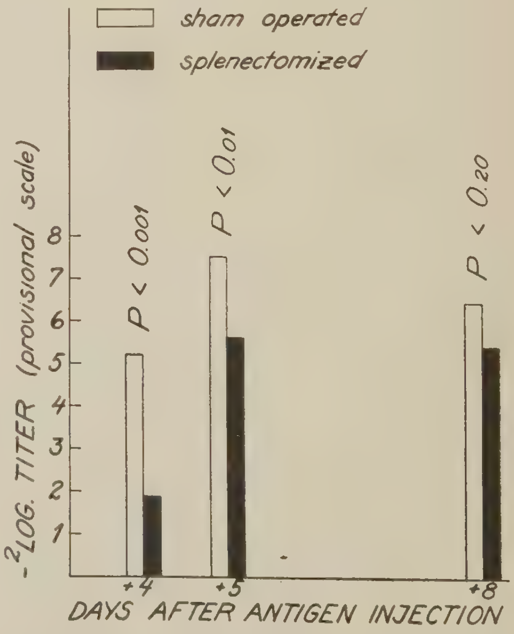

FIG. 1. Effect of splenectomy on average hemolysin response after a single intraperitoneal inj. of sheep erythrocytes. P values indicate level of significance of mean difference.

increased resistance of hemolysin production after a large intraperitoneal sheep cell dose.

Summary. 1. Splenectomy, performed one day after an intraperitoneal injection of a large dose of sheep erythrocytes, reduced the initial part of the hemolysin response, but did not significantly affect a later part. 2. The antibody-reducing effect of cortisone was significantly greater in splenectomized than in sham operated rats.

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Simplified Method for Calculating Flow, Mean Circulation Time and Downslope from Indicator-Dilution Curves. (22341)

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The use of indicator-dilution curves to measure cardiac output and "central blood volume" is widespread and of proved value (1-4). When the curves are markedly prolonged, as in heart failure, the calculations as ordinarily performed are time consuming because of the necessary integrations of the curves. Two methods of integration of the curves are in general use. The first consists of arithmetic summation of all concentrations each second from appearance time to some time later—usually through one or two cycles of semi-logarithmic paper. The second method involves replotting the curves (after extrapolation on semi-logarithmic paper) on linear paper and integrating planimetrically. The calculation of mean circulation time is done by finding the center of gravity of the linearly plotted curve on cardboard(2), or else by summing arithmetically all the products of time and concentration from the semi-logarithmic plots and dividing by the arithmetic or planimetric integration. Lewis(5), described an ingenious method of calculating the cardiac output from indicator-dilution data which eliminated the necessity for plotting the curves. Though generally useful, this method does not permit calculation of mean circulation time. In addition errors in samples which might become apparent from plots of the data could be missed more easily.

This paper proposes a method for accurately obtaining the integral, slope and mean circulation time of an indicator-dilution curve in a short time, with relatively simple calculations. The method involves a combination of arithmetic integration (summing of concentration) of a small portion of the curve, and mathematical integration of the remainder.

Method. Semilogarithmically plotted indicator-dilution curves generally can be divided into two portions. The first is the rapidly rising upslope and the curved portion of the downslope; the second is the linear portion of the downslope, extrapolated toward infinite time. It becomes apparent that the integral of the total curve is the sum of 2 areas—the area from appearance time to beginning of linearity of the downslope, and the area from the beginning of the downslope to infinite time (Fig. 1). The area of the first portion can easily be obtained by summation. Because of the steep rise and relative sharp-

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* Helen H. Millenson fellow in Cardiology Research.

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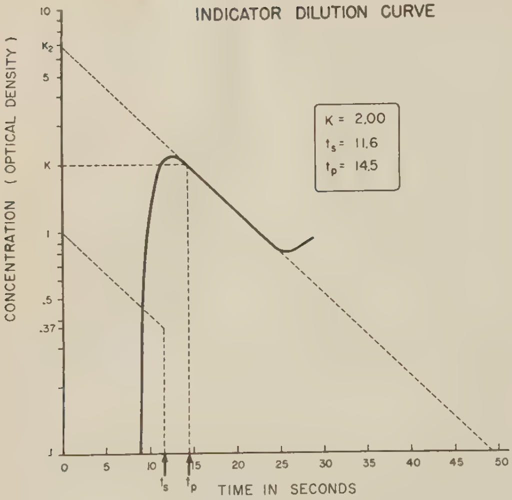

FIG. 1. Sample indicator dilution curve analyzed according to method described. (See text for symbols.)

ness of the peak only a few concentrations have to be summed—the integration of the linear portion can be determined graphically as follows, and can be added to the first integral to obtain the total area of the curve: 1. A line perpendicular to the time axis is drawn through the highest concentration on the linear downslope. The intercept of this line with the baseline is noted and recorded as t_p. 2. A line parallel to the downslope is then constructed passing through the value 1.0 of the concentration axis. Another perpendicular to the time axis is dropped from the intersection of this line with the 0.37 concentration line. The intercept of this perpendicular with the time axis is then noted and recorded as t_s. This value is the reciprocal of the downslope S and when multiplied by the flow would give the "lung" volume of Newman(4). 3. A fourth line is now drawn parallel to the time axis through the beginning of the linear downslope. The point at which this line crosses the concentration axis is noted and recorded as K. 4. The product Kt_s is equal to the integral of the straight line portion of the curve. As stated previously this integral is added to the arithmetically obtained integral of the first portion of the curve to obtain the total area. The amount of indicator injected into the circulation is divided by the total integral to obtain flow.

To calculate the mean circulation time of the entire curve we proceed as follows: 1. The product Kt_s $(t_s + t_p)$ is obtained. 2. The concentration for each second, from appearance of indicator to beginning of linear downslope, is obtained from the curve and multiplied by its respective time. These products are summed. 3. This sum is added to the value obtained from step 1. above. 4. The final sum (from 3. above) is divided by the previously obtained total area of the curve. The result is the mean circulation time, T_m. The validity of the method is apparent from examination of the mathematical derivation in the appendix.

Summary. 1. A new graphical method for calculating flow, downslope and mean circulation time from semilogarithmic plots of indicator-dilution concentration-time curves is described. 2. The advantages of employing this method of calculation include speed and accuracy.

APPENDIX: *Mathematical Proof of Method.*

I. Slope determination of indicator dilution curves. The slope is usually obtained from formula

$$(1)\quad \text{Slope } S = \frac{\log_e C_2 - \log_e C_1}{T_2 - T_1}$$

This formula is applicable with any values of C_2 and C_1 obtained from the downslope. Since the slope of any line parallel to the downslope is the same as the downslope we can construct a line parallel to the downslope passing through the 1.0 concentration value. Slope of this line would be identical with slope of our original. We can choose any 2 values of C_2 and C_1 to determine the slope. Choosing values $C_1 = 1.0$ and $C_2 = 0.37 = e^{-1}$, the corresponding times for these concentration values would be T_1 and T_2. Since our line intersects the concentration axis at 1.0 at time 0 then $T_1 = 0$. Substituting into our formula (1) we obtain

$$(2)\quad \text{Slope } S = \frac{\log_e C_2 - \log_e 1.0}{T_2 - 0}$$

Since $\log_e 1.0 = 0$ then

$$(3)\quad S = \frac{\log_e C_2}{T_2}$$

Since we have set $C_2 = e^{-1}$ we now substitute this and get

$$(4)\ S = \frac{\log_e e^{-1}}{T_2}$$

Since $\log_e e^{-1} = -1$ then

$$(5)\ S = \frac{-1}{T_2} \quad \text{or}$$

(6) $T_2 = \frac{-1}{S}$, the minus sign indicating the direction of the slope.

Thus time T_2 (the time corresponding to a C_2 of 0.37) is equal to reciprocal of the downslope.

II. Flow determination

Total area of the curve can be represented by the integral $\int_0^{\infty} Cdt$. By drawing a line perpendicular to the time axis through highest portion of the linear downslope, curve can be divided into 2 portions whose total area is the sum of the area up to the perpendicular line and the area from the perpendicular to infinite time. Expressed mathematically:

$$\int_0^{\infty} Cdt = \int_0^{t_p} Cdt + \int_{t_p}^{\infty} Cdt$$

Value of the integral $\int_0^{t_p} Cdt$ is obtained arithmetically by summation.

The value of the integral $\int_{t_p}^{\infty} Cdt$ can be shown to be K/S as follows:

If a line parallel to the downslope is drawn through the concentration axis at K, (highest value of linear downslope) then the area under this line must be equal to area under downslope since both triangular areas (on semilogarithmic paper) are congruent and at the same concentration level. The equation representing this constructed line is

$$(7)\ C = Ke^{-St}$$

its area is

$$(8)\ \text{Area} = \int_0^{\infty} Ke^{-St}dt = \text{area under downslope} = \int_{t_p}^{\infty} Cdt$$

Since K is constant we can write

$$(9)\ \int_{t_p}^{\infty} Cdt = K \int_0^{\infty} e^{-St}dt$$

Integrating the right hand expression we get

$$(10)\ \int_0^{\infty} Cdt = K\left[\frac{-1}{S} e^{-St}\right]_0^{\infty} = K/S$$

By adding this area K/S to the arithmetically obtained value of $\int_0^{t_p} Cdt$, we obtain the value of the total integral to infinite time. By dividing the amount of indicator injected, Q, by this integral the flow results from the formula

$$(11)\ \text{Flow} = \frac{Q}{\int_0^{\infty} Cdt}$$

III. Mean circulation time determination.

The mean circulation time is defined as

$$(12)\ T_m = \frac{\int_0^{\infty} Ctdt}{\int_0^{\infty} Cdt}$$

Value of denominator is determined as described above. Value of numerator is derived as follows:

The integral $\int_0^{\infty} Ctdt$ can be divided into 2 parts (by a line drawn through the highest point on the downslope perpendicular to the time axis) so that

$$(13)\ \int_0^{\infty} Ctdt = \int_0^{t_p} Ctdt + \int_{t_p}^{\infty} Ctdt$$

Value of $\int_0^{t_p} Ctdt$ is obtained arithmetically by summing all the products of concentrations and time each second up to t_p. To this sum is added value of integral $\int_{t_p}^{\infty} Ctdt$ determined as follows:

Since the curve of concentrations against time plotted semilogarithmically is a straight line from t_p to infinity this curve can be expressed by the formula

$$(14) \quad C = K_2 e^{-St}$$

where K_2 is the intercept of this line extrapolated back to the concentration axis (zero time).

Substituting (14) into the integral we are trying to determine we can say

$$(15) \quad \int_{t_p}^{\infty} Ctdt = \int_{t_p}^{\infty} tK_2e^{-St}dt$$

Since K_2 is a constant we can write

$$(16) \quad \int_{t_p}^{\infty} Ctdt = K_2 \int_{t_p}^{\infty} te^{-St}dt$$

Solving we get

$$(17) \quad \int_{t_p}^{\infty} Ctdt = K_2 \left[\frac{e^{-St}}{(-S)^2} (-St-1) \right]_{t_p}^{\infty} = K_2e^{-St_p} (St_p + 1)$$

The expression can be simplified as follows:

The area under the downslope from t_p to infinity is equal to the area under the curve obtained by drawing a parallel line through the concentration axis (zero time) at K. Equating these areas we get

$$(18) \quad \int_{t_p}^{\infty} K_2e^{-St} = \int_{0}^{\infty} Ke^{-St}$$

and solving

$$(19) \quad K_2 e^{-St_p} = \frac{K}{S}$$

or

$$(20) \quad K_2 = \frac{K}{e^{-St_p}}$$

substituting the value for K_2 from (20) into equation (17) we get

$$(21) \quad \int_{t_p}^{\infty} Ctdt = \frac{K}{S^2} (1 + St_p) = \frac{K}{S} \frac{1}{S} (1 + St_p)$$

$$\text{Since } \frac{1}{S} = t_s$$

$$(22) \quad \int_{t_p}^{\infty} Ctdt = Kt_s (t_s + t_p)$$

Adding this to the arithmetically obtained value for $\int_{0}^{t_p} Ctdt$ we get the entire value for $\int_{0}^{\infty} Ctdt$ which when substituted into equation (12) gives mean circulation time.

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Demonstration of a Toxic Factor(s) in Thermal Death.* (22342)

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Although fluid balance may play an important role in the problem of shock and thermal death, other investigations have supported the theory that toxic substances are released into the circulatory system after scalding and that this toxic material is responsible for death of the organism(1-3). Helderweirt *et al.*(4), experimenting with heat death in rabbits, noted that even when lowered blood volume of scalded rabbits is experimentally returned to normal, the organism succumbs to "another trouble still unknown." The work of LaFontaine(5) and Simonart(6,7) indicates the importance of a toxic factor in blood of scalded frogs. Although the precise function of toxic substances is still argued, it has been suggested that the toxic factor is in some way connected with protoplasmic and blood clotting(8-10).

The arguments for and against the toxic factor theory have, however, been based on experiments involving only vertebrate organisms. It is, therefore, the purpose of this study to determine if a toxic substance could be found in marine invertebrates. Although preliminary experiments uncovered several invertebrate organisms which appeared to release a toxic substance when scalded (for example, starfish), this paper will deal solely with an investigation on the marine worm, *Phascolosoma gouldii.* One can easily obtain fairly large quantities of coelomic fluid from this thick-walled, unsegmented annelid. Another advantage in using this animal is that it does not undergo autotamy when subjected to scalding, a problem frequently encountered when experimenting with many other invertebrates.

Materials and methods. In the experiments to be described, both male and female worms *(Phascolosoma gouldii)* ranging from 3-5 inches in length were used. When scalding was necessary, the posterior half of the animal was immersed for 90 seconds in hot sea water (76 ± 1°C). Following this treatment the animals were immediately returned to finger bowls containing sea water and maintained at a relatively constant temperature by stacking the bowls on sea water tables. The water within the finger bowls was changed at least once daily. In those experiments involving dialysis, dialysis membrane having a flat diameter of 1¼6 inches was washed both inside and out, and the samples dialyzed against large volumes of running sea water (approximately 500 ml per minute) for 12 hours. The end point used in determining the survival time of worms is probably subject to an error of at least ± 2-3 hours. These organisms were considered dead when pinching them with a forceps no longer produced a response such as muscular contractions. Unless otherwise stated, in all experiments a minimum of 15 animals was used to determine average survival time. Eggs of the sea urchin, *Arbacia punctulata,* were obtained by electrical stimulation, as described by Harvey(11). The eggs were washed several times in sea water and fertilized by addition of diluted sperm. The per cent cleavage was determined by counting a minimum of 100 eggs per dish.

Results. Suggestive data for the release of a toxic substance was first obtained when comparing the survival time of scalded worms to that of worms ligated prior to scalding. Results indicated that, although scalded animals would die in about 21 hours, the survival time could be increased merely by securing a ligature (a heavy thread or Hoffman clamp) just anterior to the heated region, prior to scalding (Table I). It can be seen that by preventing the coelomic fluid in the scalded portion of the animal from mixing with the anterior portion, the survival time was increased. The fact that survival time could

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TABLE I. Effect of Pre-Scalding Ligature on Average Survival Time (in Hr ± S.D.) of Scalded Worms. Posterior half of organism immersed in sea water at 76°C for 90 sec.

Non-constricted animal		Animal constricted by ligature	
Scalded	Control	Scalded	Control
21 ± 13	Infinite	74 ± 29	120 ± 79

not be increased any longer than 3- or 4-fold may be explained on the basis of a leak in the ligature, but more likely by the fact that the procedure of ligating is in itself sufficient to cause death in 120 hours.

In an attempt to isolate any toxic material that was present in scalded worms, coelomic fluid was removed from worms 15 minutes after scalding by piercing the thick muscular wall with an 18 gauge hypodermic needle attached to a 5 ml syringe. The coelomic fluid was then centrifuged and 1 ml aliquots of the supernatant injected cephalad into the posterior end of normal recipient worms. As shown in Table II, coelomic fluid from scalded animals proved toxic when injected into normal recipients. The controls, when injected with 1 ml of cell-free coelomic fluid obtained from unheated *Phascolosoma,* lived indefinitely. It appears, then, that the act of scalding releases a toxic substance which is found in the coelomic fluid and is capable of causing death when injected into normal animals of the same species.

Origin of the toxic factor. Although the toxic substance is found in the coelomic fluid, the question arises as to whether the toxin is released from coelomic fluid itself or if it originates from another source. In an attempt to answer this question, experiments were carried out in which the coelomic fluid was removed from normal worms, the coelomic cavity flushed out (with sea water) and finally refilled with sea water. This "sea-water containing" worm was then scalded and the fluid within the coelomic cavity removed, centrifuged and 1 ml aliquots injected into normal recipient worms. The results obtained from this type of experiment showed that toxin was not released in those scalded animals where sea water was injected into the coelomic cavity in place of coelomic fluid. Such experiments suggest that the toxic substance originates in the coelomic fluid. However, the possibility still exists that, in order for the toxin to be released, an inter-action may be necessary between the coelomic fluid and the rest of the worm. In the following experiments the coelomic fluid from normal animals was removed, placed in test tubes and immersed in a water bath at 76°C for 90 seconds (2 minutes in instances where the diameter of the tubes was substantially larger than the diameter of worms). This heated coelomic fluid was cooled, centrifuged and 1 ml aliquots of the supernatant injected into normal worms. The results show that the toxic factor can be released merely by heating coelomic fluid *in vitro* since those animals receiving injections of this fluid succumb in about 40 hours. (Controls receiving 1 ml injections of unheated coelomic fluid lived indefinitely.) It appears, then that the toxin can be released without any interaction between coelomic fluid and the rest of the organism. Additional experiments were carried out in which pieces of body wall, as well as viscera, were heated *in vitro* in the presence of small volumes of sea water; but the supernatant, when injected into recipient worms, proved non-toxic.

TABLE II. Effect of Cell-Free Coelomic Fluid Taken from Scalded Worms on Average Survival Time (in Hr ± S.D.) of Normal Worms.

Worms inj. with coelomic fluid from scalded donor	Worms inj. with coelomic fluid from normal donor
29 ± 15	Infinite

By separating coelomic fluid into two portions, (1) the cells of the coelomic fluid—which were re-suspended in sea water and (2) the cell-free supernatant, it was shown, after heating at 76°C, that the toxic factor was released only in that fraction of coelomic fluid containing the cells. Thus the origin of the toxic substance released in this marine annelid after scalding appears to be in the cells of the coelomic fluid. It might be brought out at this point that preliminary experiments suggest that the toxin can be released by treatment other than heat—particularly homogenization.

Characteristics of toxic factor. The series of results, tabulated in Table III, show that

TABLE III. Effect of Heat and Dialysis on Toxic Factor Activity. Average survival time (in hr ± S.D.) of normal worms receiving 1 ml injections of precipitate-free coelomic fluid.

Cell-free coelomic fluid of scalded worm		Cell-free coelomic fluid of normal worm	
Autoclaved	Autoclaved & dialyzed	Autoclaved	Autoclaved & dialyzed
32 ± 14*	77 ± 32	Infinite*	Infinite

* Only 9 worms injected.

the toxic factor is still active after autoclaving (15 lb pressure per square inch). This heat-stable toxin appears to be a relatively large molecule (or at least tied up with a large molecule) in that after prolonged dialysis against running sea water, the toxic fraction still proves fatal when injected into normal recipients (Table III).

Experiments have shown that the toxic factor was not ether-soluble since ether extracts of toxic coelomic fluid, after having been evaporated and redissolved in sea water, had no apparent effect when injected into normal worms. The ether-insoluble portion still exhibited its toxicity. Although the toxic coelomic fluid has a lower pH than does the normal (normal = pH 7.8; toxic = 6.7), readjusting the hydrogen ion concentration, followed by injection of normal worms, shows that the toxicity was not due to any pH change. In like manner, it was shown that the toxicity was not due to any potassium differences that may be present between experimental and control coelomic fluids.

In an attempt to further isolate the toxic factor, samples of the coelomic fluid were heated *in vitro,* centrifuged and the supernatant autoclaved. The protein coagulated by autoclaving was then centrifuged off and the supernatant then saturated with ammonium sulfate. This resulted in the appearance of a flocculent precipitate. These fractions were separated by centrifugation and the precipitate redissolved in sea water (a volume equal to that of the supernatant). The 2 fractions, the supernatant and the precipitate, were then dialyzed until all traces of ammonium sulfate had been removed. When 1 ml aliquots of these fractions were injected into normal worms (Table IV) the fraction containing the precipitate, which was "salted out" by saturation with ammonium sulfate, proved toxic.

It might be noticed, when comparing the data presented in Tables II, III and IV, that animals injected with samples of coelomic fluid that have been dialyzed survive over twice as long as those animals injected with non-dialyzed coelomic fluid. These data suggest the possibility that two types of toxic molecules are released after scalding—one dialyzable and the other non-dialyzable.

Semi-micro assay. Since the only method of assay has been that of injecting fluid into recipient worms, a procedure requiring large volumes of toxin, it was felt that a micro assay was necessary, particularly if chromatographic studies were to be pursued. Eggs of the sea urchin, *Arbacia punctulata,* are relatively sensitive to the toxic factor, for when these eggs were fertilized in sea water and 5 minutes later immersed in the toxic fraction, cell division was substantially inhibited. It has been shown that only 6% of the eggs undergo cleavage when immersed in a 6.25% solution of cell-free coelomic fluid taken from scalded worms (diluted in sea water). Further proof that the factor inhibiting cell division is the same factor responsible for the toxic effects in the worm is seen in experiments in which fertilized *Arbacia* eggs were immersed in the fraction precipitated by ammonium sulfate (similar to those used in Table IV). As can be seen in Table V, cell division was completely inhibited in such instances although the various controls underwent substantial cleavage.

Summary. In a study of heat death, it has been shown that the marine annelid, *Phascolosoma gouldii,* releases a heat stable, non-

TABLE IV. Isolation of Toxic Factor by "Salting Out" with $(NH_4)_2SO_4$. Assayed by determining average survival time (in hr ± S.D.) of worms receiving 1 ml injections. Coelomic fluids were partially purified prior to saturation with $(NH_4)_2SO_4$ by autoclaving.

Partially purified toxic coelomic fluid saturated with $(NH_4)_2SO_4$		Partially purified normal coelomic fluid saturated with $(NH_4)_2SO_4$	
Precipitate (dialyzed)	Supernatant (dialyzed)	Precipitate (dialyzed)	Supernatant (dialyzed)
56 ± 13	Infinite	Infinite*	Infinite

* Only 8 worms injected.

TABLE V. Effect of Toxic Factor, Isolated by "Salting Out" with $(NH_4)_2SO_4$, on Cleavage of *Arbacia punctulata* Eggs. Coelomic fluids were partially purified by autoclaving, followed by centrifugation and dialysis.

	Partially purified toxic coelomic fluid saturated with $(NH_4)_2SO_4$		Partially purified normal coelomic fluid saturated with $(NH_4)_2SO_4$		
	Precipitate	Supernatant	Precipitate	Supernatant	Sea water control
	(Dialyzed)				
% of eggs cleaved	0	97	94	96	99

dialyzable toxic factor, capable of causing death when injected into normal worms. This toxic substance, originated from the cells of the coelomic fluid, has been partially isolated by saturation with ammonium sulfate. A semi-microbiological assay has been described.

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Reduction of Mitotic Activity in Pinna Epidermis of Mice Given Cortisol or 9α-fluorocortisol.*† (22343)

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The inhibitory effect of cortisone upon mitotic activity in certain tissues seems to be established(1-4). In this study, antimitotic effects were noted for hydrocortisone (cortisol) and for 9α-fluorocortisol. In order to detect antimitotic effects occurring within the range of the daily mitotic rhythm, this work was carried out 1) under environmental conditions standardized for evaluation of 24-hour periodicity(5), 2) during the period of day of relatively high mitotic activity, 3) on a population of mice previously found to exhibit a marked mitotic rhythm in pinna epidermis(6).

Materials and methods. For 7 days prior to experiment, male ZBC mice, 8 weeks of age, were kept singly housed in a room maintained at 80 ± 1°F, illuminated from 6 A.M. to 6 P.M., and kept dark from 6 P.M. to 6 A.M. Purina Fox Chow and tap water were available to the mice *ad libitum*, from the time of weaning and throughout experiment. On the day of experiment, a first group of 11 mice was not handled, while 3 other groups, each composed of 12 mice, were given intraperitoneally 100 γ or 1,000 γ of cortisol acetate or 10 γ of 9α-fluorocortisol acetate. Each mouse was weighed prior to injection and the dose was adjusted to refer to 20 g of body weight. The treatments were given in rotation, in order to minimize the possible role of a time factor difference among treatments.

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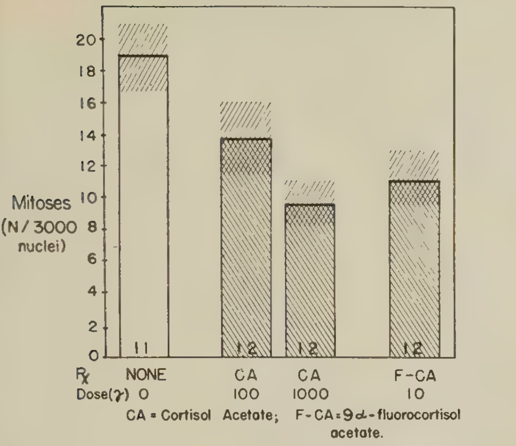

FIG. 1. Effects of cortisol and 9 α-fluorocortisol upon pinna mitoses of intact male ZBC mice (the height of the columns denotes the means, the shaded areas denote ± 1.0 S. E.).

TABLE I. Significance of Inter-Group Differences in Mean Mitotic Counts.

Difference studied*		DF	Difference* ± S.E.	t	P
9 α-fluorocortisol acetate	10 γ	21	7.7 ± 2.6	2.94	.008
Cortisol acetate	1000 γ	21	9.1 ± 2.3	3.90	<.001
” ”	100 γ	21	4.8 ± 2.9	1.65	.115

* Mean mitotic count of "no treatment" group *minus* the corresponding value for treatment group listed.

The injections were made from 8:30 A.M. to 9:30 A.M. of the same morning, and the injected and uninjected mice were all sacrificed 4 hours later. The ears were clipped off by means of scissors, the margins were trimmed, and the ears were then placed into Bouin's fixative. The time elapsed from the moment of removal of a mouse from its cage until the immersion of its ear into the fixative did not exceed 10 seconds. Following embedding in paraffin, at least 20 sections of each ear were prepared. The sections, about 6 μ thick, were stained with Heidenhain's iron hematoxylin and were counterstained with eosin. In sections from each of the ears, 3,000 epidermal cells were examined under oil immersion and the total number of mitoses as well as their respective stages were recorded.

Results. The height of the columns in Fig. 1 shows the mean mitotic count for each of the groups studied, the shaded areas denoting ± one standard error. It may be seen that the mice given corticosteroid treatments had lower mean counts, as compared with untreated mice. As far as cortisol treatment is concerned, the group receiving 1000 γ had a lower mean count than that receiving 100 γ. Furthermore, the antimitotic effect of 10 γ of 9α-fluorocortisol compared favorably with the corresponding effect of 100 γ and unfavorably with that of 1000 γ of cortisol. The effects of both 1000 γ of cortisol and 10 γ of 9α-fluorocortisol were significant at the one percent level, as may be seen from Table I. The proportions of the 4 mitotic stages in the groups receiving either 10 γ of 9α-fluorocortisol or 1000 γ of cortisol were, however, roughly comparable to those in the untreated group, as shown in Table II.

The χ^2's for the slight differences in proportions of mitotic stages between the untreated group on one hand and the 10 γ of 9α-fluorocortisol and 1000 γ of cortisol groups, on the other hand, were 2.25 ($P > .39$) and 1.89 ($P > .57$), respectively. By contrast, the corresponding difference between the untreated group and the 100 γ of cortisol group was significant below the 5% level ($\chi^2 = 8.53$; $P = .037$). The latter group differs from the former groups primarily by a higher proportion of prophases and by a lower proportion of telophases. This difference in proportions may have been brought about by the shorter duration of the antimitotic effect of the lower dose of cortisol. Conceivably, in mice given 100 γ of cortisol, at 4 hours post-injection, cells may have entered prophase without inhibition, but as yet, they may not have ad-

TABLE II. Distribution of Mitotic Stages in Groups Investigated.

Treatment	Dose (γ)	% prophase	% metaphase	% anaphase	% telophase
None		26	43	11	20
9 α-fluorocortisol acetate	10	26	37	11	26
Cortisol acetate	1000	22	39	16	23
” ”	100	33	44	13	10

vanced to telophase (Table II). This possibility, however, can not be supported without additional data obtained at earlier times after injection.

The main points of this report are the following: First, the administration of cortisol in 1000 γ doses, as well as that of 9α-fluorocortisol in 10 γ doses, results in a significant reduction in number of mitoses in pinna epidermis. Second, doses of the compounds which exhibit significant antimitotic effects do not change significantly the proportions of mitotic stages. Accordingly, these compounds may not interfere with the mitotic process *per se*, but rather, they may act upon some aspect of interphase. Third, a comparison of those doses of cortisol and of 9α-fluorocortisol which exhibit antimitotic activity supports the suggestion that the substitution of a fluorine atom in the 9α position results in a marked enhancement of the antimitotic activity of cortisol. The fluorine-substituted compound has more than 10 times the activity of cortisol, under the conditions of this study. This suggestion is in keeping with the enhancement of other biologic activities of cortisol by fluorine-substitution in the 9α position(7-13).

Summary. Mitotic activity in pinna epidermis of male ZBC mice was studied under environmental conditions standardized for evaluation of 24-hour periodicity, during the period of day of relatively high mitotic activity. Under these circumstances, antimitotic effects, exerted apparently during interphase, were noted for hydrocortisone and for 9α-fluorohydrocortisone. The latter compound had more than 10 times the antimitotic potency of the former.

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Plasma Enzyme Actvity in Myocardial Infarction in Dog and Man.* (22344)

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Increases of plasma transaminase(1) and lactic acid dehydrogenase in liver disease(2) and myocardial infarction(3) suggested the probability of the release of other enzymes from the myocardium or the liver as a result of hypoxia or tissue necrosis. This laboratory has been interested in the immediate changes following experimental myocardial infarction in dogs. For example, a higher concentration of pyruvate in coronary sinus than in arterial blood (negative myocardial balance of pyruvate) was observed within the first thirty minutes following coronary embolization(4). This phenomenon has also been observed in hemorrhagic shock(5) and in ventricular fibrillation(4). It is likely that the reduction in coronary flow which immediately follows

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embolization of the coronary arteries is responsible for the metabolic changes. Furthermore, myocardial ischemia leads to necrosis, with concomitant changes in cell permeability and loss of enzymes into the blood. The present report is concerned with variations in the plasma levels of transaminase, isomerase, aldolase, and malic acid dehydrogenase in dogs with experimental coronary occlusion and in man suffering from myocardial infarction.

Methods. Pyruvate was determined by the method of Friedemann and Haugen(6). Experimental coronary occlusion was produced by following essentially the method of Agress (7). However, human albumin was substituted for gum acacia as a vehicle for suspension of the plastic spheres (size of spheres: 325 mu; amount injected: 2 mg/kg weight). This modification was necessary since it was found that gum acacia alone can produce infarction with typical pathologic and biochemical alterations. Plasma transaminase activity was determined by the method of Karmen *et al.*(1) and plasma aldolase by that of Bruns (8); the latter is expressed as micrograms of triose phosphate formed per ml of plasma per hour at 38°C. Plasma isomerase activity was determined by the method of Bodansky(9) expressed in activity units as therein described. The determination of malic acid dehydrogenase in the presence of other unidentified enzymes and metabolites presents considerable difficulties. The reaction of malate, in the presence of DPN and malic acid dehydrogenase comes to a rapid standstill presumably due to accumulation of oxalacetic acid. The addition of glutamate, in the presence of excess transaminase removes this difficulty(10). Since, however, the reaction is greatly in favor of oxalacetate ⟶ malate(9) and involves only a few components, it was tested as follows: To 0.1 - 0.05 ml of plasma was added 2 ml of 0.1 M phosphate buffer pH 7.55 and 0.1 ml DPNH$_2$ (2.5 mg/ml); the volume was made up to 3.0 ml with distilled water. After 10-15 minutes 0.1 ml of 0.5 M oxalacetic acid (Sigma) at pH 7.0 in 0.1 M phosphate buffer was added. The change in optical density at λ340 of DPNH$_2$ was observed for 3 to 4 30-second periods to assay the reaction rate. The oxalacetate solution was prepared just before use and was kept in a glass stoppered test tube in ice water. In addition to decarboxylation reactions(11) which would result in some lactic acid dehydrogenase activity being measured simultaneously, oxalacetate can enter many

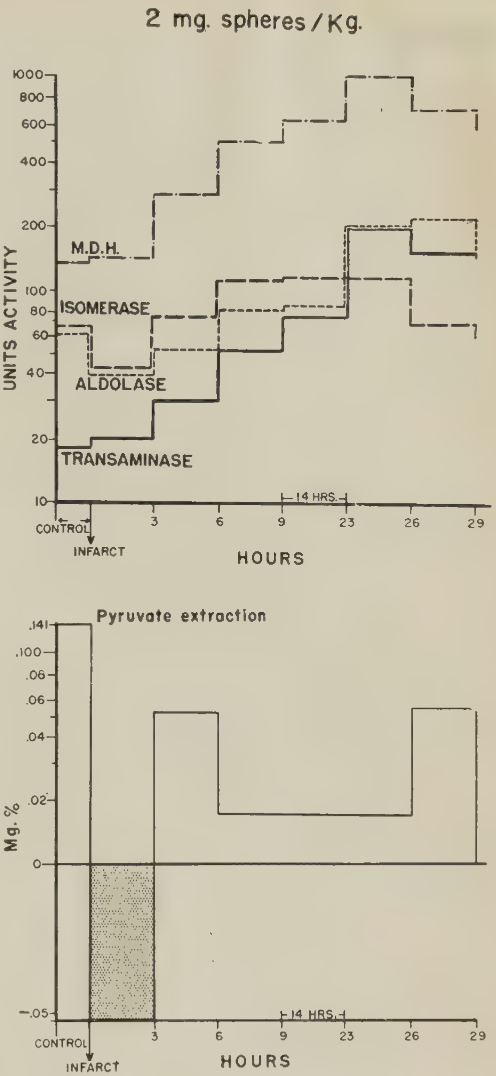

FIG. 1. Variations in plasma activity of malic acid dehydrogenase, isomerase, aldolase, and transaminase. Changes in plasma enzyme activity are correlated with myocardial pyruvate extraction. It may be seen that there is an immediate drop in myocardial pyruvate extraction; however, the negative myocardial balance is of short duration. Enzyme activity in plasma commences to rise 3 hr after coronary embolization. Peaks in plasma enzyme activity occur 24 hr following embolization.

other pathways(12). While a sharply rising activity with pH has been reported for lactic acid dehydrogenase(2) with a maximum at pH 9.0, the reaction described has an almost constant activity between pH 7.5 and 8.1. The range of normal activity units and the standard deviation are also less than that reported for lactic acid dehydrogenase(3). Since the reaction may not be entirely specific for malic acid dehydrogenase, it will be referred to in quotation marks.

Results. The average, range and standard deviation for the plasma concentrations of these enzymes in normal dogs are as follows: transaminase, 23, (10-37), 9; "malic acid dehydrogenase", 108, (42-195), 47; isomerase, 33, (16-68), 17; aldolase, 42, (24-68), 18.

Fig. 1 is representative of the results obtained after coronary embolization. In a series of 9 experimental animals, 7 were examined and showed postmortem evidence of myocardial infarction. Four animals of the experimental group were subjected to coronary sinus catheterization and showed negative myocardial pyruvate balances. In 3 of these animals myocardial pyruvate balances were negative within 15 minutes after the infarction and then became positive (Fig. 1). One showed a negative balance at 3 hours. It is seen from Fig. 1 that the significant release of enzymes began by the third hour. At 24 hours the increase in enzyme activity was maximal for all animals. At that time, the coronary flow and the coronary vascular resistance which had been diminished immediately following embolization of the coronary arteries had returned to their control levels. At 48 hours a rapid fall in plasma enzyme activity ensued. After 24 and 48 hours both transaminase and "malic acid dehydrogenase" showed increases of comparable degree, with aldolase and isomerase exhibiting increases of a lesser order.

Table I gives the normal values on a small series of humans without hepatic or cardiac disease. In man the average value, range and standard deviation for "malic acid dehydrogenase" and isomerase were less than those seen in the dog. Results obtained in patients with myocardial infarction are also illustrated in Table I. As compared to the dog, aldolase activity was least elevated in patients with myocardial infarction. Isomerase activity in plasma increased following in general the trend of transaminase. Similar findings were obtained with "malic acid dehydrogenase."

The high activity of "malic acid dehydrogenase" in normal plasma suggests the possi-

TABLE I. Levels of Plasma Transaminase, Malic Acid Dehydrogenase and Isomerase in Normals and Patients with Myocardial Infarcts.

		Transaminase	Malic acid dehydrogenase	Isomerase* (Bodansky units)	Aldolase as triose-phosphate formed per ml plasma per hr
Normals	Avg	25 (9)	79 (7)	13 (10)	26 (7)
	Range	15–39	50–104	6–19	21–32
	Stand. dev.	8	20	4	5
L.J.	2/ 5	185	333	111	59
	2/ 6	150	200	30	32
	2/ 7	47	163	15	25
	2/ 8	37	120	12	21
V.A.B.	2/ 6	155	300	51	41
	2/ 7	95	208	20	32
P.E.	1/30	123	200	94	65
P.P.	2/11	100	647	374	39
J.P.	2/11	34	240	54	24
	2/13	62	110	7	24
	2/14	43	152	9	24

* Normal values reported on a series of 87 patients by Bodansky: Avg, 20; range, 8–40; stand. dev., 8.

Figure in parenthesis represents No. of animals.

bility that the transaminase reaction could be run adequately without the addition of the expensive and labile malic acid dehydrogenase enzyme preparation. Omitting this enzyme has little effect on the results of the determinations on stored dog plasma, when the transaminase levels were at the limit or beyond the established normal range. However, when transaminase activity is determined in stored human plasma, the omission of malic acid dehydrogenase leads to results which are low and inconsistent.

The system used to determine "malic acid dehydrogenase" activity by means of the rate of oxidation of DPNH in the presence of oxalacetic acid and plasma may not measure malic acid dehydrogenase activity exclusively. Nevertheless, the rate of $DPNH_2$ oxidation appears to be closely related to the increase of activity of other enzymes, which are observed following myocardial infarction. The reaction appears to be particularly useful because of its simplicity and rapidity. Similarly, the isomerase determinations in myocardial infarction may be of clinical importance since they can be carried out without specialized equipment.

Summary. 1. Increased plasma levels of transaminase as well as aldolase, isomerase and "malic acid dehydrogenase" activity occurred in dogs after experimental myocardial infarction and in humans suffering from coronary occlusion. 2. Plasma levels of all enzymes were highest 24 hours after experimental myocardial embolization and had fallen rapidly at 48 hours. 3. Normal human values for plasma isomerase and "malic acid dehydrogenase" were significantly lower than those found in normal dog plasma. 4. In dogs following experimental myocardial embolization plasma transaminase, "malic acid dehydrogenase" and aldolase were increased to a greater extent than isomerase. In humans the plasma aldolase activity was comparatively less elevated than that of transaminase, "malic acid dehydrogenase" and isomerase. In both man and dog, coronary occlusion resulted in comparable increases of plasma transaminase and "malic acid dehydrogenase" activity. In dogs with coronary embolism, there was a reversible decrease in coronary flow myocardial oxygen usage, coronary vascular resistance as well as an early reversible negative pyruvate balance. At 24 hours the coronary flow and myocardial oxygen usage had returned to normal. 5. The negative myocardial pyruvate balance appeared to be an immediate response of an hypoxic myocardium. In contrast, the release of enzymes into the plasma was correlated with muscle necrosis. 6. A rapid and simple method is described for the determination of "malic acid dehydrogenase" activity in plasma.

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Metabolism of Acetoacetate-3-C^{14} by Mammalian Heart Muscle. (22345)

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In a previous paper(1) we have presented evidence indicating that the net increase of cardiac glycogen observed in ketonemic animals(2) does not represent a direct conversion of ketone bodies *per se* to carbohydrate. That demonstration invalidates the most obvious interpretation which can be applied to the data of Lackey *et al.*(2), and relegates the ketonemia to some secondary role yet to be defined. While our data was direct and conclusive, the subject is one of sufficient importance to require evaluation from a different viewpoint before definitely ruling out the possibility that cardiac tissue may have some unique metabolic mechanism for dealing with ketone bodies.

In the present paper we have used the isolated dog heart to examine the metabolic path traversed by acetoacetate-3-C^{14} as reflected by the isotope distribution in the endogenous malate and succinate isolated at the end of the experiment to serve as an internal metabolic indicator. Glycogen is frequently employed in this capacity(3) but was valueless here because no tagging of that molecule occurs under the conditions used, an observation which is consistent with our *in vivo* experiments(2).

Materials and methods. The isolated dog heart was used in a simple glass apparatus containing the usual components and through which the heparinized blood (500 ml in all experiments) was circulated by means of a manual "rubber bulb-check valve" type of pump. The gas outlet from this closed system was led through 2 refluxing setups for trapping acetone in boiling acid mercuric sulfate (4) and thence to an efficient alkali CO_2 absorbing tower. A slight vacuum was used to overcome the mild resistance to the free flow of gas through the system. *Sodium acetoacetate-3-C^{14}* was synthesized and prepared by established methods(5). Five millimoles of the radioactive compound was introduced dropwise into the flowing blood just as it entered the marble filled gassing tower. The 500 ml of circulating fluid was thus 0.01 M with respect to acetoacetate. Our experiment made use of several different gases: 100% O_2, 95% O_2-5% CO_2 and, in a few instances, a gas mixture calculated to simulate the oxygen tension found *in situ* and composed of 19% O_2-5% CO_2-76% N_2. With the first 2 gases we used undiluted blood in the apparatus and obtained a very satisfactory heart preparation. With the latter mixture however, the blood did not appear well oxygenated and quickly assumed a marked viscosity with resultant limitation in coronary blood flow, and feeble heart action. In these instances we diluted the blood 1:1 with Ringer-bicarbonate in order to maintain an adequate fluid flow. Even then the preparation was not nearly as vigorous as at the higher oxygen levels. At the end of a 2 hour experimental period, 2 slightly different approaches were used to isolate the malate and succinate present in the heart tissue. In one instance we followed directly the procedure outlined by Needham(6) after adding 0.2 mM of succinate or malate carrier. In the other we introduced more drastic conditions to assure release of any tissue bound dicarboxylic acids, or those that might perhaps be tied up in some sort of ester linkage. Using a Waring Blender, the cardiac tissue was homogenized in ice water (carrier succinate added) and made to pH 2 with concentrated sulfuric acid. The entire material was steam distilled, collecting ten volumes of distillate, and the resulting tissue suspension reduced to an almost dry paste at mild temperatures using a blast of dry air. After adding concentrated sulfuric acid and dehydrating with plaster of Paris the succinate was recovered and purified(6). Occasionally we effected further purification by use of partition chromatography on silicic acid(7). Our results were not affected by the method of extraction. Carbon-wise degrada-

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TABLE I. Isotope Distribution in Malate and Succinate following Metabolism of Sodium Acetoacetate-3-C^{14} by Isolated Dog Heart.

Gas	Other additions	Total C^{14} added,* cts/min. (×1000)	$C^{14}O_2$ in respiratory CO_2†	% substrate to CO_2	Specific activity, malate cts/min./mg/C		Specific activity, succinate	
					Carboxyl C	Methylene C	Carboxyl C	Methylene C
O_2	None	2,626	703,000	26.7	683	0		
19% O_2–5% CO_2–76% N_2	”	4,150	791,420	19	2,553	17	1,748	58
Idem	Adrenalin	4,150	337,777	8	2,026	10		

* As sodium acetoacetate-3-C^{14}.

† Blood acidified at end of experiment and bound CO_2 aerated using N_2.

tion of the isolated succinate or malate (and occasionally both) was effected by established methods(8) giving the activity respectively in the carboxyl and methylene carbons.

Results. According to current concepts, acetoacetate-3-C^{14} is metabolized by first splitting into 2 "acetate" molecules one of which, in this case, would be labeled in the carboxyl group. Further breakdown would be effected by traversing the tricarboxylic acid cycle and producing among the various other components, succinate and malate labeled exclusively in the carboxyl groups(3). For each "acetate" entering the cycle 2 carbons are lost as CO_2 and this mechanism therefore cannot account for a net increase in any component. If the results of Lackey *et al.*(2) represent a direct relation between ketone bodies and net carbohydrate synthesis, then a path not involving the tricarboxylic acid cycle must be invoked. This could happen, for example, if omega oxidation converted acetoacetate directly to oxalacetate and thence to glucose via pyruvate. In this instance 3 of the 4 initial carbons would be available for the net synthesis. Another possibility involves the reduction of acetoacetate to butyrate and thence to succinate via omega oxidation and finally to glucose as in the above case. The latter is unlikely in view of Lorber's(9) recent demonstration that butyrate is metabolized only in the conventional manner, an observation which we have confirmed in the isolated dog heart(10). If either of the latter mechanisms is operating on acetoacetate-3-C^{14} the endogenous malate and succinate would be tagged in the methylene carbons.

Table I gives data which are typical of our various experiments. Only comparison between the activity in the carboxyl *vs* methylene carbons should be made. The absolute specific activity of the several samples is not comparable because the amount of carrier dicarboxylic acid added to aid in the isolation procedure varied according to circumstances. It is obvious that in comparison to the carboxyl groups, the methylene carbons of the malate and succinate contain an insignificant amount of C^{14}. This is consistent with the view that acetoacetate is being metabolized only in the established manner, *viz*, by breakdown to two two-carbon fragments which then traverse the tricarboxylic acid cycle(3), a path which cannot effect a net increase of any component.

We have made additional observations that seem worth recording. We were curious to assay the possibility that the heart may contain dormant alternate metabolic paths on which it can draw in an emergency. Accordingly, we stimulated a preparation to abnormally vigorous activity by the periodic addition of adrenalin to the circulating blood. As shown in Table I the isotope distribution in malate was not altered. Whether the low total conversion to $C^{14}O_2$ (8%) is a function of the adrenalin or merely a reflection of variability from preparation to preparation is a matter under consideration.

Summary. The metabolic path of acetoacetate-3-C^{14} in the isolated dog heart has been determined using the isotopic distribution in the endogenous malate and succinate as an internal metabolic indicator. Isotopic carbon was found exclusively in the carboxyl groups of malate and succinate as would be expected if acetoacetate was being utilized only by the established path (acetoacetate → 2 "acetate" → 4 CO$_2$ via the tricarboxylic acid cycle). This is taken as corroborative evidence for our previous conclusion that ketone bodies *per se* are not responsible for the net increase in cardiac glycogen noted in ketonemia.

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Effect on Cardiac Glycogen of Intravenously Administered Sodium Acetoacetate-3-C^{14}. (22346)

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In dogs made diabetic by pancreatectomy the glycogen content of liver and skeletal muscle decreases while that of heart increases (1). A similar pattern has been observed in rats injected with large amounts of acetoacetate or B-hydroxybutyrate(2). It becomes a matter of some importance to establish whether the ketone bodies *per se* are being metabolized in a manner directly responsible for this net increase in cardiac glycogen or are merely acting in some secondary role. In the former instance a new and yet unrecognized mechanism would have to be operating, since the conventional metabolic path (acetoacetate → 2 "acetate" → 4CO$_2$ via the tricarboxylic acid cycle) is not compatible with a net increase in any component. If ketone bodies on the other hand are acting only in some indirect manner, that role too must be defined. The data published by Lackey *et al.* (2) simply notes the quantitative increase of cardiac glycogen without throwing any light on possible mechanisms. The present paper is the first in a series designed to define this particular metabolic area. Here we have made the direct test of whether ketone bodies *per se* go to heart glycogen. We have injected into rats large amounts of acetoacetate-3-C^{14} and subsequently isolated the cardiac glycogen to determine the quantity and distribution of C^{14} in the glucose molecule. By this means strong inferences concerning the metabolic path can be obtained.

Materials and methods. Sodium acetoacetate-3-C^{14} was synthesized according to established methods(3) and put into a solution at pH 7.4 such that per ml there was 0.88 mM (11%) of acetoacetate containing an amount of C^{14} giving 600,000 cts/min. The quantity of isotopic material available limited the study to 3 rats. The nature of the results is such that valid conclusions are clearly possible despite the small number of animals involved. A 200 g rat, warmed by a heat lamp, was anesthetized with nembutal, the jugular vein exposed, and the fine cannula of an intravenous drip apparatus introduced. In each of our 3 experiments the gravity flow ceased after about 2½ hours at which time 3 ml (2.6 mM and 1,800,000 cts/min.) of the acetoacetate solution had been introduced. In one rat we used a hypodermic syringe to inject an additional 2 ml into the tail vein over a period of half an hour. In all instances a total of 4 hours elapsed before the animal was sacrificed. Following this, the heart was removed, washed with saline and dropped into hot 30% KOH together with 35 mg of purified glycogen made from rat liver. The carrier material was necessary in view of the fact that a single rat heart contains only about 2 mg of glycogen which is an inadequate amount for isolation, purification and degradation. The dilution does not interfere with evaluation of radioactivity since counting was done on the total sample which was highly purified (4), degraded to CO_2 by persulfate oxidation (5) and assayed as $BaC^{14}O_3$.

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Results. According to Lackey *et al.*(2) the infusion of acetoacetate can cause as much as a 60% increase in heart carbohydrate. In the average rat this represents formation of approximately 1.5 mg of glycogen over and above that normally present. We would not expect our experiments to effect this much synthesis since we introduced only about 60% as much ketone as the original workers. However, assuming that we obtained only 10% as much synthesis and assuming that no more than one C^{14} from the acetoacetate was incorporated into each glucose of the glycogen molecule, we would have expected radioactivity in the order of 500 counts per minute. In none of our experiments did the glycogen molecule contain any traces of radioactivity. We are forced to conclude therefore, that the increase in heart glycogen accompanying ketonemia does not represent a direct conversion of ketone bodies to carbohydrate. The mechanism of this effect is still an open question.

Summary. The intravenous injection of large amounts of acetoacetate-3-C^{14} has been effected under conditions reported to cause a net increase in heart glycogen. Isolation and degradation of the cardiac glycogen failed to divulge any trace of radioactivity. It thus appears that the net increase in heart carbohydrate accompanying ketonemia represents some secondary and yet undetermined influence of ketone bodies.

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Feasibility and Safety of Frequent Plasmapheresis of the Same Human Donors.* (22347)

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(Introduced by T. N. Harris.)

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Plasmapheresis is the obtaining of whole blood from a donor with the retention of the plasma; the red blood cells being returned to the donor. This has been accomplished in the past in both man and other animals by well known technics(1-5). These are laborious, time consuming, and have many other obvious drawbacks inherent in the methods which would prevent large scale use. The present study is concerned with plasmapheresis in man utilizing the ADL-Cohn Blood

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Fractionator.[†] The theoretical aspects of this technic have been discussed(6). With this equipment the red blood cells and plasma are continuously separated during the phlebotomy. It provides a sterile closed mechanical system for taking blood from a donor and separating it into many of its constituents, any of which may be returned immediately to the donor through the same needle used for the phlebotomy. The temperature of the removed blood during separation is decreased to 8°C or lower in a very short period of time which prevents adverse enzyme activity. Completely non-wettable surfaces have been utilized throughout, consisting of either polyvinyl plastic[‡] or silicone-coated[§] metal. A complete description of the Blood Fractionator is being prepared(7). One of the problems being investigated in the present study is to determine whether biweekly plasmapheresis of the same donor over a period of one year is safe and feasible. This project has been in progress since April 1955 (about 40 weeks) and because of the practical applications, as well as the broad theoretical implications, it seemed advisable to present a preliminary report.

Methods and materials. Plasmapheresis technic. The donor lies on a table placed on a raised platform which, by gravity, increases the rate of flow of blood to the Fractionator which is about 40 inches lower. The phlebotomy is started with the insertion of a silicone-coated 15 gauge needle into a larger vein, usually in the antecubital fossa. The blood flows through a 3 way stop cock[||] and through an ion exchange resin column[¶] which removes the calcium and some of the platelets. The decalcified blood then continues into the centrifuge bowl, which for this operation is designed to accept a fixed volume of 500 ml of fluid for each run. While the bowl is spinning, only the plasma (the lighter component) is allowed to flow from the bowl into a plastic bag. No admixture of red blood cells occurs. The red blood cells, which are heavier, accumulate within the bowl and flow by gravity, after the bowl is stopped, into another plastic bag. The plastic tubings, through which the plasma and red blood cells respectively flow to the bags and which are connected to the bowl are then sealed[**] (a one-half inch wide seal), and the seal is cut through the middle with a pair of scissors. This operation thus produces sterile containers of plasma and red blood cells with short lengths of attached tubing sealed at the end. Red blood cells are returned to the donor by connecting the plastic tubing attached to the red cell bag, to the 3 way stop cock. Incorporated in the plastic tubing outlet of the red cell bag is a drip chamber and a nylon mesh filter. In the 3 to 4 minute period during which the red cells drop from the centrifuge into the bag, sterile physiological saline is slowly infused into the donor in order to keep the lumen of the needle and tubing open for the return of the red blood cells. To some extent, before the red cells are returned, the saline replaces temporarily the volume of the whole blood removed. *Donors.* Twenty-three men are being utilized in this study. Nineteen of these volunteers are members of the Philadelphia Park Police between the ages of 26 and 35, in excellent physical condition. The remaining 4 men are between 37 and 43 years of age. *Tests.* The following determinations are made at each visit: Blood pressure, pulse, red blood cell count, white blood cell count, differential counts, platelet count, hemoglobin, hematocrit, sedimentation rate and prothrombin clotting time. In addition, paper electrophoretic pattern and nitrogen content are determined for each plasma.

Results. Table I shows that there is very little variation in the red cell count, hemoglobin, hematocrit and plasma nitrogen in a single donor subjected to biweekly plasmapheresis over a period of 40 weeks. In addition the amount of time for each phlebotomy and red blood cell return is recorded. These data, pertaining to a single donor, are representative of the entire group of 23 individuals in the study.

White blood cell counts, differential counts,

[†] Mfd. by A. D. Little, Inc.

[‡] Mfd. by Fenwal Labs., Inc.

[§] Mfd. by General Electric.

[||] The 3 way stop cock is being replaced by a plastic Y tube.

[¶] Dowex 50.

[**] Mfd. by Scientific Specialties Corp.

TABLE I. Effect of Biweekly Plasmapheresis on the Same Donor over a Period of 40 Weeks.

	Weeks										
	0	4	8	12	16	20	24	28	32	36	40
Red blood cell counts ($\times 10^6$)	4.7	4.9	4.6	4.5	5.6	5.2	4.9	5.4	5.1	4.7	4.9
Hemoglobin (g)	15.8	15.2	15.1	16.3	15.4	15.0	15.7	15.0	15.8	14.9	14.5
Hematocrit (%)	42	40	41	38	40	40	40	43	45	43	46
Plasma N (%)	1.07	1.14	1.00	1.02	1.08	1.00	1.03	1.01	1.04	1.08	1.06
Phlebotomy time*	7 35	9 45	9 00	7 40	7 46	10 02	7 21	7 40	15 10	7 23	7 40
R.B.C. return time*	7 50	10 10	9 12	12 35	9 40	7 05	8 30	9 08	11 12	10 57	9 34

* First number in these columns is minutes, last 2 numbers are seconds; *e.g.*, 7 35 = 7 min. 35 sec.

Figures on alternate biweekly plasmaphereses are omitted because of lack of space.

platelet counts, sedimentation rates and prothrombin clotting times carried out routinely at each donor's visit have been quite constant, much the same as would be found in any normal person.

To make this technic a practical procedure, the time consumed must be relatively short. The average time required for the phlebotomy and red blood cell return of the 23 men for 20 plasmaphereses (a total of 460) is $8' 50''$ and $10' 04''$ respectively. Allowing 3 minutes for the red cells to drop after the centrifuge has been stopped, the total time elapsed is $8' 50'' + 10' 04'' + 3' =$ about 22 minutes. It is a simple matter to run 3 complete plasmaphereses per hour by beginning a phlebotomy on the second donor after the red blood cells are started in return to the first donor.

Over 550 plasmaphereses have been carried out since the start of this project including the preliminary and trial runs. No untoward reactions have been observed in any donor.

Discussion. The utilization of the new equipment mentioned has made frequent plasmapheresis of the same human donors both safe and feasible. This permits the use of donors for plasma only, with the replacement of their red blood cells, thus allowing 26 bleedings per year in this study. Fifty plasma donations yearly should be perfectly feasible since the human plasma proteins are regenerated rapidly(1,3), and since a very small percentage of the total plasma proteins is removed at one time. (200 ml plasma x 7.5% protein equals 15 g of protein out of a total plasma protein content of about 250 g, or about a 6% removal at one time.)

If 10 times the customary number of donations can be obtained from a single individual, it should be possible to achieve large savings in the collection of plasma since only one-tenth the number of donor centers would be required.

By plasmapheresis, single plasmas, as free of bacteria and hepatitis virus as the usual single donation of whole blood, can be preserved in non-porous bags, easily stacked and non-breakable, which can be left at any desired temperature for the inactivation of serum hepatitis virus. This virus is probably present in 0.5 to 1% of individual bleedings from the general population, but with selected donors for repeated plasmapheresis the incidence of virus in single plasmas should be considerably less than in whole blood. The possibility of untoward reaction in the recipient, to certain plasmas could be obviated by testing the individual plasma before use.

The plasmapheresis technic makes feasible the use of human donors in the preparation of high titered specific antibodies for therapy. The danger of foreign protein reaction is practically eliminated. Furthermore, it has been found that, unit for unit, the ratio of effectiveness of human antibody is far superior to horse antibody(8). As part of the current project the persistence of viral and bacterial antibodies in hyperimmune and other donors undergoing biweekly plasmapheresis is being determined. A further use of this method would be to obtain relatively large amounts of plasma from individuals who have rare antibodies for a short period of time, *e.g.* mothers following delivery, who have certain Rh or Hr antibodies which decrease in titer fairly rapidly.

Summary. Biweekly plasmapheresis on the same human donors over a period of 40 weeks has been found to be feasible and safe. The possibilities for obtaining large amounts of concentrated human antibodies and for stockpiling sterile human plasmas are discussed.

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Failure of Ovarian Hormones to Maintain Pregnancy in Rats Deficient in Pantothenic or Pteroylglutamic Acid.[*] (22348)

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Recent studies have shown that reproductive failure, *i.e.*, fetal death and resorption, can be prevented and pregnancy maintained by daily injections of estrone and progesterone in rats subjected to 4 different dietary deficiencies, those of thiamine(1), pyridoxine (2), protein(3,4) or potassium(5). The daily dosage levels of the hormones were the same as those found to be effective in maintaining pregnancy in rats hypophysectomized and oophorectomized after breeding(6). The present communication reports the failure of these hormones to prevent fetal death and resorption in rats deficient in either pantothenic acid(7) or pteroylglutamic acid(8,9).

Methods. The procedures for testing the efficacy of the hormones were the same as those used previously to maintain pregnancy in other dietary deficiencies(1-5). The experimental conditions necessary for each vitamin deficiency to result in fetal death and resorption in approximately 90% of the animals were determined, if not already known. The synthetic hormones were dissolved in sesame oil and given daily, either separately or in combination, by subcutaneous injection from the 3rd or 5th days of gestation period to the day before autopsy. Levels of estrone varying from 0.5 to 2.0 μg[†] daily were given separately and levels from 0.5 to 1.0 μg were given in combination with 4 mg of progesterone. Five to ten rats (Long-Evans strain) were used for each experimental group. Vaginal smears were examined daily for the presence of erythrocytes, the sign of implantation. The rats were autopsied near the end of the gestation period, from the 15th to the 21st day. The basal (control) purified diet was composed of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated vegetable oil (Crisco or Primex), and 4% salts no. 4(10). Crystalline vitamins per kilo of diet were: 300 μg d-biotin, 5 mg 2-methyl-1,4-naphthoquinone, 5 mg thiamine HCl, 5 mg pyridoxine HCl, 5.5 mg pteroylglutamic acid, 10 mg riboflavin, 10 mg p-aminobenzoic acid, 20 mg

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[†] Maintenance of pregnancy has been shown to vary with level of estrone given to protein-deficient rats(3).

TABLE I. Failure to Maintain Pregnancy by Injections of Estrone and Progesterone in Pantothenic Acid-Deficient Rats.*

Daily hormone dosage	Rats bred (No.)	Wt change during gestation (g)	Onset vaginal RBC (day)	Resorptions (%)	Litters (%)
		Pantothenic acid-deficient diet			
None	15	+ 5	12.4	79	21
Estrone 1 μg	6	+14	12.2	83	17
Progesterone 4 mg	10	+ 3	12.2	80	20
Estrone .5 μg + Progesterone 4 mg	10	− 3	12.1	80	20
		Pantothenic acid-supplemented diet			
None	10	+115	13.1	0	100

* All groups averaged 25-30 days of deficiency prior to breeding. Control group received pantothenic acid-supplemented diet only from day of breeding to autopsy (day 17-18). Hormone injections were given daily from day 5 to autopsy.

niacin, 50 mg d-calcium pantothenate, 400 mg inositol, and 1.0 g choline chloride. All rats received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vit. A, 115 chick units vit. D, 6 mg dl-alpha-tocopherol, and 650 mg corn oil (Mazola). Omission of calcium pantothenate from this diet resulted in a pantothenic acid-deficient diet similar to that used previously(7) but improved in vitamin supplementation. In the PGA-deficient diet PGA was omitted and 1% succinylsulfathiazole and 0.5% x-methyl PGA incorporated in the diet. Fifty mg PGA per kilo of diet (10 times the usual level) was added to this diet for control animals. The PGA-deficient and supplemented diets are identical with those used previously(8,9).

Results. Pantothenic acid deficiency. Normal female rats from the stock colony were placed on the pantothenic acid-deficient diet at 90 to 100 days of age and bred after 25 to 30 days of deficiency. Hormone injections were given from the 5th day of gestation to the 17th or 18th days, when all animals were autopsied. Table I demonstrates the failure to maintain pregnancy by estrone and

TABLE II. Failure to Maintain Pregnancy by Injections of Estrone and Progesterone in Rats Subjected to Pteroylglutamic Acid (PGA) Deficiency.*

Daily hormone dosage	Rats bred (No.)	Wt change during gestation (g)	Onset vaginal RBC (day)	Resorptions (%)	Litters (%)
		PGA-deficient diet instituted day of breeding			
None	10	+15	12.5	100	0
Estrone 1 μg	5	+31	13.0	100	0
Progesterone 4 mg	5	+14	12.8	100	0
Estrone 1 μg + Progesterone 4 mg	5	+32	12.8	100	0
		PGA-deficient diet instituted day 9			
None	22	+62	12.8	100	0
Estrone 2 μg	10	+62	12.6	100	0
Estrone 1 μg + Progesterone 4 mg	10	+71	12.6	90	10
		PGA-supplemented diet instituted day of breeding			
None	5 21	+ 58 +112	12.0 12.8	0 0	100 100

* Hormone injections were given daily from day 3 to autopsy when PGA-deficient diet was instituted on day of breeding but were not given until day 9 in 2nd group when PGA-deficient diet started day 9.

progesterone in these deficient rats. Resorption occurred in approximately 80% of the animals, whether uninjected or given the ovarian hormones, either separately or combined. In all groups vaginal erythrocytes (RBC) appeared normally on the 12th to 13th days. The animals showed no signs of the deficiency with the exception of a failure to gain weight during the gestation period.

Pteroylglutamic acid deficiency. Stock female rats were bred at 90 to 100 days of age and placed immediately on the PGA-deficient diet. Injections of the hormones were started 3 days after breeding and all rats were autopsied on the 15th to 16th day of gestation. Table II shows the ineffectiveness of the ovarian hormones in maintaining pregnancy. Resorption occurred in all animals, regardless of the presence or absence of injected hormones. A less severe PGA-deficiency was then produced by instituting the PGA-deficient diet 9 days after breeding, a procedure previously reported to result in 100% resorptions(9). Hormone therapy was instituted at the same time as the PGA-deficient diet. Again the hormones were ineffective as fetal death and resorption occurred in 90-100% of the animals in all groups.

In all PGA-deficient rats vaginal erythrocytes occurred normally on the 12th to the 13th days and the animals exhibited no external signs of the deficiency, although the maternal weight gain during the gestation period was less than that of vitamin-supplemented controls.

Discussion. The failure to maintain pregnancy by injection of ovarian hormones in this study was in marked contrast to the successful maintenance of pregnancy with the hormones in deficiencies of thiamine, pyridoxine, protein or potassium. Under the experimental conditions reproductive failure resulting from pantothenic acid or PGA deficiency did not appear to be due to lack of production or secretion of the maternal sex hormones as has been indicated for the other four dietary deficiencies. The signs of hormonal inadequacies observed in rats subjected to the other four dietary deficiencies or in rats hypophysectomized and oophorectomized after breeding, namely early appearance of vaginal erythrocytes and extremely rapid resorption of implantation sites, were not observed in rats deficient in pantothenic acid or in PGA. There were no disturbances in the estrous cycle in unbred rats given the pantothenic acid or PGA-deficient diets for the same length of time. In this study the maternal organism was only slightly affected, few or no external signs of either deficiency syndrome being observed, even though fetal death resulted. The deleterious effects of both pantothenic acid and PGA deficiencies on embryonic development are well established as multiple congenital abnormalities have been observed in the offspring when a less severe deficiency was employed(9,11-13). In contrast, congenital abnormalities have not been reported for those deficiencies in which pregnancy can be maintained by injections of the ovarian hormones.

Summary. Daily injections of estrone and progesterone, given separately or combined, failed to prevent fetal death and resorption in rats deficient in pantothenic acid or in pteroylglutamic acid. Dosage levels of the hormones were the same as those used successfully to maintain pregnancy in rats hypophysectomized and oophorectomized after breeding as well as in rats deficient in thiamine, pyridoxine, protein or potassium. Under the experimental conditions, reproductive failure in rats subjected to pantothenic acid or PGA-deficiency did not appear to be due to lack of the maternal sex hormones.

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Improved Urinary Excretion Test for Assay of Intrinsic Factor. II. Sensitive Counting Technic.* (22349)

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The measurement of urinary excretion of vit. $B_{12}Co^{60}$ ($B_{12}Co^{60}$) following oral administration is used in the diagnosis of pernicious anemia(1,2) and as an assay for intrinsic factor(3,4). The use of human beings as experimental subjects necessitates the use of $B_{12}Co^{60}$ of low activity.† Since Co^{60} is a gamma emitter, the instrument of choice for its measurement is the scintillation counter. Most present well-type scintillation counters however, cannot count more than a 10 ml sample and therefore the low activity present in a 24 hour urine sample presents a counting problem which can only be solved by excessively long counting or by concentration of the urine samples. Flat scintillation crystals, upon which a beaker of liquid can be placed, do not offer significantly greater sensitivity than the well-type scintillation counter. Weiner and Peterson(5) designed a G-M well counter for measuring the radioactivity in liquid samples contained in 3 liter bottles, whereas Baskin *et al.*(6) constructed a well-shaped scintillation counter with a 35 ml capacity.

Using an available small well-type scintillation counter, a beaker with a capacity of one liter was designed fitting into the well and surrounding the top and sides of the crystal. This paper reports on the results obtained with the use of this beaker.

Methods. The design of the scintillation counter‡ is shown on the left side of Fig. 1. The counter consists of an RCA 5819 phototube, a thallium activated sodium iodide crystal, 1¾ in. diameter and 1 in. thick with a hole ¾ in. in diameter and 1 in. deep, and a cathode follower preamplifier. A 4 ml sample can be counted in a screw-cap glass vial and the vial is then placed in the well separated by a brass sleeve to prevent contamination of the crystal housing. The beaker in counting position is shown on the right side of Fig. 1. It was constructed§ starting with a two liter beaker from which the bottom was removed. The new bottom enclosed the crystal on top and sides with a projection fitting downward into the well. The counter and beaker were enclosed in a lead shield 3 in. thick. The background was about 110 cpm.

Results. 0.5 μc of vit. $B_{12}Co^{60}$ was diluted to 1000 ml and increasing amounts of this solution were placed in the beaker and counted. The values reported in Table I are corrected for background. As is evident from Table I, the use of the beaker gives an increase in counting rate of approximately 40 times that obtained using the standard 4 ml vial. As one would expect, the efficiency of counting with the beaker (3.5%) is less than direct counting in the vial (22%), but the over-all gain due to the larger volume is much greater than the loss in efficiency. It is reasonable to expect that much greater sensitivity would result if the beaker was designed to fit over larger crystals such as the commercially available 1¾ in. diameter by 2 in. thick crystals.

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* See Ref. 4 for Paper I in this series.

† The maximum permissible dose has been set at 3 μc by the Subcommittee on Permissible Internal Doses of the National Committee on Radiation Protection.

‡ Model S_1—Purchased from H. O. Anger, Berkeley, Calif.

§ Constructed by L. & K. Glass Co., Lyndhurst, N. J.

Due to the geometry of this beaker and crystal a volume of 500-750 ml gave the greatest efficiency, since at this volume there is equal amounts of liquid equidistant from all surfaces of the crystal. The optimum method, though, is to count one liter of liquid if available.

The increase in sensitivity permits the use of $B_{12}Co^{60}$ of lower specific activity in the urinary excretion test. One subject can receive 12 doses of 0.25 μc instead of 6 doses of 0.5 μc therefore, allowing the use of the same patient for additional intrinsic factor assays. Furthermore, the use of the beaker requires less counting time with a low excretion of radioactivity and makes it unnecessary to concentrate the urine sample. Some of the advantages of the beaker over the vial methods are listed in Table II. Even at the 5.0% excretion value if the dose of $B_{12}Co^{60}$ administered were reduced to the same amount as permitted by the use of the beaker and if the urine were not evaporated it would be necessary to count 100 minutes in order to reduce the standard error of counting to below 5%.

TABLE I. Comparison of Sensitivity of Vial *vs.* Beaker Counting Methods.

Vol	C.P.M.	Activity in sample, μc	Efficiency, %	Beaker sensitivity ratio*
Vial				
4	978	.002	22	—
Beaker				
100	3,783	.05	3.4	3.9
200	7,832	.10	3.5	8.0
300	12,695	.15	3.8	13.0
500	21,769	.25	3.9	22.2
750	32,492	.375	3.9	33.3
1000	38,666	.50	3.5	39.8

$$* \ \frac{\text{Efficiency} \times \text{volume of beaker}}{\text{Efficiency } (22\%) \times \text{volume of vial } (4 \text{ ml})}.$$

The technic described in this report also appears applicable to other biological materials of large volume and low activity from gamma radiation. It would seem particularly suitable for counting $B_{12}Co^{60}$ in feces in

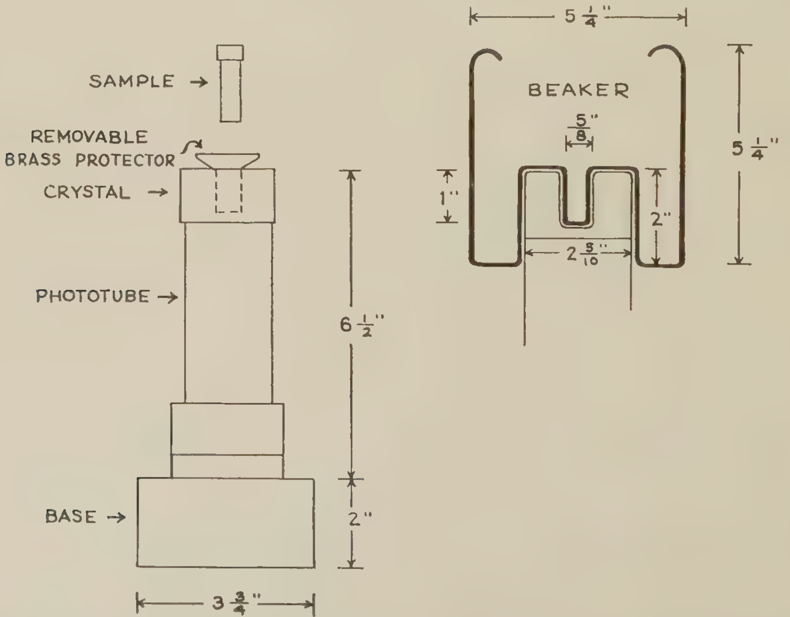

FIG. 1. Left: Well-type scintillation counter for measuring gamma radiation contained in a 4 ml glass vial. Right: Same counter showing design and position of a beaker for counting up to one liter of liquid.

TABLE II. Comparative Features of Vial and Beaker Counting Methods.

Excretion in 24 hr urine sample[*]	0.5%		5.0%	
	Vial	Beaker	Vial	Beaker
Dose of $B_{12}Co^{60}$ administered (μc)	.5	.25	.5	.25
Max permissible No. of doses/patient	6	12	6	12
Degree of concentration	10	None	10	None
Vol counted (ml)	4	1000	4	1000
C.P.M. corrected for background	49	97	490	970
Counting time[†] (min.)	20	9	1	1

[*] Assuming the 24 hr urinary vol to be 1 liter.

[†] Minimum necessary time so as not to exceed 5% stand. error of counting and assuming a constant background rate of 110 cpm.

the fecal excretion technic for the estimation of intrinsic factor activity(7).

Summary. Measurement of gamma radiation of low specific activity contained in relatively large volumes can be adequately performed with the use of a specially designed beaker which fits into the well and covers the cross section of a well-type scintillation counter. The increase in sensitivity obtained with this beaker allows the administration of smaller oral doses of vit. $B_{12}Co^{60}$ for additional assays using the same patient for the urinary excretion tests for intrinsic factor. In addition, no concentration of the urine sample is necessary and less counting time is required.

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Effect of Nalorphine on Gastric Emptying in Man. (22350)

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The consensus of most authorities is that morphine noticeably retards the emptying of the stomach in the human(1-3), as well as in the dog(4). Nalorphine, which antagonizes the narcotic effect of morphine when both are present, has certain actions when given by itself similar to morphine, but less intense. It is, therefore, of interest to determine the effect of nalorphine alone on gastric emptying.

Methods. The experiments were performed on 7 healthy medical students, 5 males and 2 females. The test meal consisted of 15 g of farina, cooked to a volume of 200 cc. One hundred g of barium sulfate were added so that the meal could be seen with the fluoroscope. The subjects ate this meal about 8:30 A.M. No food had been eaten since the previous evening. Determinations were made at weekly intervals. Fifteen minutes before they ate the meal, the subjects were given a subcutaneous injection of either saline (controls) or 5 mg of nalorphine hydrochloride[*] in saline. The time it took the meal to leave the stomach was determined fluoroscopically to the nearest 15 minutes.

Results. Table I shows that nalorphine delayed gastric emptying in all subjects, and more than 4 hours in 2. Only two showed a delay of less than 2 hours. All subjects, ex-

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[*] We wish to thank Merck and Co. for the gift of nalorphine hydrochloride used.

TABLE I. Effect of Nalorphine on Gastric Emptying Time of 7 Subjects.

Controls		Experimental			
No. of trials	Avg emptying time, hr	No. of trials	Avg emptying time, hr	Diff.	p*
3	2.67	3	6.83	4.16	.03
4	2.44	3	5.17	2.73	<.01
4	2.25	3	2.83	.58	.10
4	2.06	4	2.81	.75	.05
3	2.08	3	6.42	4.34	.02
3	2.00	2	5.25	3.25	.09
3	3.08	3	6.00	2.92	.01
Avg	2.37		5.04	2.67	<.01

* p according to the Fisher t test.

cept No. 3 and 6, showed a statistically significant delay, and in No. 6 lack of significance is possibly due to the small number of trials since he showed a large difference. The average delay for the series as a whole is significant at the one per cent level.

Discussion. Initially, a dose of 10 mg was tried on 4 of the subjects, but this produced severe malaise, and since at the end of 4 hours practically none of the meal had left the stomach, the experiment was discontinued. The dosage was reduced to 5 mg for all subsequent work, and the results reported here are based entirely on this dose.

According to a recent textbook of pharmacology(5), the effects of nalorphine in man, when morphine or other narcotic has not been given previously, are similar to those of morphine but less intense. The delay in gastric emptying reported here is at least as great as that produced by morphine, and possibly greater. There is some disagreement among various workers about the magnitude of the morphine effect on the stomach(6).

Summary. Five mg of nalorphine administered subcutaneously caused an average delay of over 2½ hours in the emptying time of the stomach of 7 human subjects.

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Effect of Growth Hormone and Testosterone on Induction of Cardiovascular Changes in Choline-Deficient Rats.* (22351)

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Severe choline deficiency may induce fatty and necrotic changes in cardiac muscle fibres, medial coronary lipoidosis and medial sclerosis of the Moenckeberg type in aortas of rats fed a high fat hypolipotropic diet(1-3). It is not known whether those changes are primarily due to choline deficiency or are secondary phenomena associated with the haemorrhagic renal cortical necrosis (HKL). Evidence in favor of the latter explanation is accumulating.

We have found that growth hormone and testosterone administered either alone or together to choline-deficient rats greatly enhances occurrence of HKL in animals which are in a borderline state of choline deficiency. Cardiovascular disease is increased in hormone-treated animals.

Methods. In a pilot experiment using 80 animals, Hall and Bieri's findings(4) were confirmed and borderline conditions for our final experiment were delineated. This re-

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† Research Fellow of Canadian Life Ins. Officers' Assn.

TABLE I. Composition of Diet WCA.

Casein	7
Peanut meal*	28
Soya protein†	5
Salt mixture‡	3
Sucrose vit. mixture‡	1
Sucrose	11
Starch	10
Lard	34
α-tocopherol acetate§	.015
Cod liver oil‖	.01
Corn oil§	1

* Extracted with 50%, 75% and 95% ethanol.

† Water washed ''alpha proteins'' (Glidden Co.).

‡ For details see: *Canad. J. Med. Sci.*, 1953, v31, 135.

§ Fat-soluble vitamins dissolved in corn oil so that 1% of dietary corn oil will supply the desired amount of fat-soluble vitamins.

‖ Obtained from Ayerst McKenna and Harrison, Montreal; contains 200,000 i.u. vit. A and 50,000 i.u. vit. D/g.

port deals only with this final experiment in which 44 female rats were used. Using our high-fat severely choline-deficient diet, it was found that weight range between 110 and 115 g in female rats was just suitable, at a room temperature of 72 degrees, for production of the desired borderline conditions in control animals. All animals were put on the same choline-deficient diet, the composition of which is shown in Table I. One experimental group was injected with 3 mg of growth hormone subcutaneously once daily; another with 1 mg testosterone (CIBA) subcutaneously once daily, while a third group was injected with both hormones. Choline-deficient animals injected with saline served as controls. The growth hormone used was obtained from the Connaught Laboratories in Toronto, lot 200, 1955 and was prepared by the method described by Campbell and Davidson(5). It contains less than 0.05 US unit TSH, less than 0.005 US unit ACTH and less than 0.2 US unit Prolactin.

The animals were injected for a maximum of 14 days. The survivors in the experimental groups were then kept another 3 weeks on a choline-deficient diet, to detect any late effect of the treatment. They were then sacrificed at the same time as the saline-injected controls.

Results. Table II illustrates the effect of growth hormone and testosterone administered either alone or together to young female choline-deficient rats. The saline-injected females all had fatty livers but the incidence of kidney and heart lesions was very low. There were no deaths from bilateral renal cortical necrosis in the control group. All control animals which showed slight involvement of the kidney due to lack of choline ("renal frosting," Hartroft(6)) were able to sustain a critical period of kidney damage between the 10th and 14th experimental day. Fatty infiltration of cardiac muscle fibres and focal cardiac necrosis were present in mild degree. The aortas of the saline-injected controls did not exhibit any lesions.

All the animals given growth hormone, however, showed a marked degree of kidney and cardiovascular involvement. The livers were fatty. More than half died from acute bilateral haemorrhagic renal cortical necrosis. The survivors in the growth hormone group had advanced degrees of 'renal frosting' as is shown in Table II. The rate and severity of cardiovascular involvement increased with the advent of HKL. At least half the aortas showed the Moenckeberg-type lesion described elsewhere(1).

The findings in the testosterone-injected animals were similar but slightly less advanced. The animals injected with both growth hormone and testosterone exhibited more severe lesions but not more frequently than the animals given growth hormone

TABLE II. Effect of Growth Hormone and Testosterone on Cardiovascular Changes in Choline Deficiency.

				Pathology					
				Kidney			C. V. system		
Group	Avg wt, g	Avg food intake, g/day	No. of rats	Frosting	HKL	Total	Cardiac	Aortic	Total
Control	112	14.5	18	6	0	6	3	0	3
Growth hormone	112	14.3	7	3	4	7	4	3	7
Testosterone	110	11	9	3	6	9	6	3	6
Growth hormone + testosterone	117	12.6	10	3	7	10	5	4	5

alone. The number of animals used does not permit an accurate assessment of the effects of the different hormones. We were primarily interested in elucidating the aetiology of cardiovascular disease in choline deficiency and less so in the ability of different hormones to aggravate it.

The average daily food consumption was calculated for the first week of the experiment only. Thereafter, the experimental animals exhibited loss of weight, reduction and irregularity of food intake. The hormone-injected animals perhaps showed a slight decrease in their food intake in comparison with the saline-injected controls. (Table II).

Discussion. The observation of Hall and Bieri(4), on the aggravating effect of growth hormone on choline deficiency has been confirmed and extended. Under suitable conditions of borderline choline deficiency, growth hormone may force animals into the full-blown picture of severe bilateral renal cortical necrosis (HKL). With the development of HKL, cardiovascular changes make their appearance in increased frequency and severity. The results of our present growth hormone experiment provide further evidence(11) that the appearance of cardiac lipoidosis and necrosis, coronary medial lipoidosis and the Moenckeberg-type of arteriosclerosis in choline deficiency are probably closely associated with HKL. It is probable that growth hormone in our experiments stimulates protein synthesis(7). This would augment the methionine requirement, which in turn would increase the severity of choline deficiency, as less methionine would be available to act as a precursor of choline. The increased severity of choline deficiency may then aggravate the bilateral renal cortical necrosis and this lesion may play its part in the cardiovascular changes. This concept is supported by the fact that results similar to those obtained with growth hormone are secured by testosterone administration. Testosterone may be a powerful protein anabolic hormone(8) in the presence of insulin(9).

Growth hormone did not increase the food intake in our choline-deficient animals. It increases appetite when administered to normal animals, but this effect was not demonstrable in choline deficiency.

Selye(10) was able to produce cardiovascular changes (collagen disease like) by growth hormone and/or DCA injection in rats which were "sensitized" to these two hormones. The nature of the lesions produced does not resemble closely those which we have found. (Moenckeberg type).

Summary. 1. The work of Hall and Bieri on aggravation of choline deficiency by growth hormone has been confirmed. 2. Under suitable borderline conditions, growth hormone precipitates haemorrhagic lesions of the kidney in choline-deficient animals. With the advent of kidney lesions, cardiovascular changes appear. 3. These findings provide further evidence that the aetiology of cardiovascular lesions in choline deficiency is closely associated with kidney damage, but a causal relationship of one lesion to the other can not be established by experiments of this type. 4. Testosterone has effects similar to those of growth hormone in choline deficiency. 5. The mechanism of this aggravation of choline deficiency has been briefly discussed.

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Inhibition of Estradiol-Induced Pituitary Hypertrophy in Rats.* (22352)

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If adult rats are treated chronically with estrogens, their pituitary glands undergo changes in morphology and activity(1-4). Several natural and synthetic estrogens produce increases in weights of hypophyses and adrenals, along with other changes. Adrenal hyperplasia is apparently related to stimulation of ACTH production. This has been shown directly by bio-assay of circulating ACTH in blood of estrogen treated rats(5) and by indirect evidence. Increase in adrenal size and adrenal ascorbic acid depletion occur following estrogen treatment of intact rats(6, 7) but not in hypophysectomized rats given estrogens(8,9). Since stimulation of ACTH production and pituitary enlargement are usual consequences of prolonged estrogen treatment it seems of interest to determine if there is a causal relationship between morphologic and physiologic effects.

Synthetic estrogen tri-*p*-anisyl chloroethylene (TACE) has been reported as of no effect on pituitary size following administration of large doses for long periods of time(10). Including this compound, we compared several estrogens for their effects on pituitary weight and ACTH secretion in rats. Certain combinations were administered with total estrogenic dose adjusted so that comparisons could be made with groups receiving equi-potent doses of a single estrogen.

Methods. Dose groups of 16 male rats of Sprague-Dawley strain, weighing approximately 160 g at start of experiment, were employed. Estradiol-17β, diethylstilbestrol, and TACE were dissolved in olive oil so that each 0.1 cc contained 10 rat units. Estradiol-17β and diethylstilbestrol were administered subcutaneously. TACE was administered orally rather than subcutaneously because its mg potency is greater by this route. Estrogens were given daily for 30 days, alone and in combination in doses indicated in Table I. When combinations of estrogens were used, each was administered at a separate site. Control animals received olive oil orally and subcutaneously in volumes equal to those employed in experimental groups. Animals were sacrificed by decapitation on day following administration of last dose, and the pituitary, adrenals, thymus, and testes removed, blotted dry, and weighed on torsion balances.

Results. Estradiol or stilbestrol administration resulted in significant increase of pituitary weights, adrenal hyperplasia, and involution of the thymus glands. No pituitary hypertrophy occurred with TACE; but the effects on adrenal and thymus sizes were comparable to those obtained with other estrogens. Simultaneous administration of TACE with estradiol prevented pituitary enlargement, obtained when the same amount of estradiol is given alone. There was no significant inhibition of the estradiol-induced hypertrophy by concurrent injection of stilbestrol. However, contrary to the additive effect one might expect, a pituitary average weight resulted, lower than obtained when only estradiol is administered. The same inhibitory effect occurred when the two estrogens used in combination were stilbestrol and TACE. Combinations of 2 estrogens were neither additive nor inhibitory in regard to adrenal and thymus weight changes. Since estrogens produce adrenal hypertrophy and thymus involution only in rats with intact hypophyses, it is assumed that these estrogen effects are indirect, depending on stimulation of ACTH release from the hypophyses. Testicular atrophy, indicative of prolonged estrogenic suppression of pituitary gonadotrophin production, was approximately the same in all groups.

Discussion. That stilbestrol causes a significant, but lesser degree of pituitary hyperplasia than estradiol, and that TACE causes no increase in pituitary weight should first be considered on the basis of comparative

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* Supported in part by grant from National Science Foundation to the State University of Iowa.

TABLE I. Average Body and Organ Weights following 30 Days Treatment with Estrogens.

Groups*	Daily dose, R.U.†	Final body wt, g	Avg pituitary wt, mg	Avg adrenal wt, mg	Avg thymus wt, mg	Avg testis wt, mg
Control		230	7.4 ± .4‡	14.9 ± .6	351 ± 20	1367 ± 40
TACE	10	190	7.3 ± .4	23.2 ± 1.2	257 ± 14	645 ± 70
	20	183	7.7 ± .1	23.8 ± 1.7	232 ± 21	784 ± 95
Stilbestrol	10	203	9.1 ± .3	28.2 ± 1.0	291 ± 19	684 ± 62
	20	198	9.1 ± .4	27.1 ± 1.2	237 ± 11	730 ± 71
Estradiol	10	203	15.6 ± 1.1	28.0 ± 1.1	247 ± 18	805 ± 79
	20	209	20.6 ± 1.2	26.9 ± 1.7	225 ± 14	841 ± 74
Estradiol + TACE	10 20 10	185	9.9 ± .6	29.2 ± 4.3	264 ± 23	642 ± 53
Estradiol + Stilbestrol	10 20 10	198	12.5 ± 1.3	34.0 ± 1.1	215 ± 15	627 ± 55
Stilbestrol + TACE	10 20 10	196	8.2 ± .8	23.3 ± 1.2	254 ± 15	788 ± 76

* 16 animals/dose group.
† 10 R.U. = Estradiol, 3.2 μg; stilbestrol, 30 μg; TACE, 210 μg.
‡ ± stand. error.

doses of each estrogen used. In the design of our experiment equi-potent doses of each estrogen were approximated using a biologic criterion of activity (the rat unit), *i.e.*, the dose necessary to cause vaginal cornification within 72 hours in 50% of a group of castrated female rats. However, even this endpoint cannot serve to equate the doses precisely since duration of effect for each estrogen differs. Estradiol and stilbestrol could be termed short-acting estrogens, since vaginal cornification following a single dose rapidly disappears. On the other hand, TACE is long-acting, maintaining vaginal cornification for several days following a single administration. In this 30-day study, a difference of this nature would tend to "favor" the short-acting estrogens because the 30 daily injections would create a series of sharp peaks and drops in circulating estrogen level, whereas a more constant estrogen stimulus to the pituitary follows similar treatment with TACE. It cannot be supposed that the constancy of estrogen levels, *per se*, is the variable factor responsible for pituitary-sparing effect, since parabiosis experiments show that endogenous estradiol stimulation from enlarged ovaries of a parabiont united to a castrate partner brings about substantial pituitary enlargement(11).

Pituitary changes following estrogen combination treatments warrant special consideration. In every case, the average pituitary weight following administration of 2 estrogens fell between the averages obtained when each substance was given individually. The most striking inhibition, therefore, occurred when TACE, which causes no pituitary enlargement, was administered concurrently with estradiol, which creates the largest pituitaries. These results suggest a competitive action between the 2 estrogens, rather than an additive one as occurs when the dose of estradiol is doubled.

In addition to pituitary hypertrophy during estradiol or stilbestrol treatment, greater ACTH activity is indicated by adrenal weight increase and thymus involution. But TACE treated animals, with normal sized pituitaries, also appear to undergo some measure of adrenal stimulation. This observation suggests that estrogen-induced pituitary hyperplasia and increase of ACTH output are not necessarily related phenomena. Substantiating this view are the data showing that even though it was possible to inhibit pituitary weight increases by combining TACE with another estrogen, no inhibition of adrenal stimulation occurred in this situation.

Final consideration should be given to the question of non-specific stress caused by estrogen treatment. When estrogens are administered to rats, starvation is a possible evocative agent for adrenal changes(12), since lowered food intake is a characteristic occurrence. Thus, adrenal effects, symptomatic of increased ACTH output may result from estrogen stimulus directly, or may be caused indirectly by hunger situation created. In the present experiment, interpretation of results does not hinge on this matter since the problem under consideration is the relationship between estrogen-induced pituitary hypertrophy and ACTH output, regardless of whether the latter is directly or indirectly stimulated. Furthermore, major comparisons of adrenal effects are made among groups which have all been treated with estrogens so that food deprivation factor is constant. Food consumption values are approximately the same for each group. However, this factor bears on the problem of expressing data as average absolute gland weights or gland weights per unit body weight, for the final body weights of experimental groups were less than controls. In our results, the relationships described are in the same direction on either basis but since group differences are somewhat altered by different treatment of data a choice between the two may be pertinent. It is our belief that the data expressed as average gland weight express more precisely the actual trend of events since body weight loss of estrogen treated groups, particularly the TACE groups, is due mainly to loss of body fat. It presents a somewhat artificial analysis to adjust glandular weights according to depletion of body fat stores.

Summary. Equi-potent doses of estradiol caused greater pituitary enlargement in rats than stilbestrol. A third estrogen, TACE, caused no enlargement whatsoever. When two estrogens were given concurrently there was a competitive, rather than an additive, action. Hence, with each combination the average pituitary weight was between averages obtained when each substance was administered individually. With all estrogens there was evidence for ACTH-stimulated adrenal hyperactivity, determined by increase in adrenal weight and thymus gland involution. The observations that (1) TACE causes adrenal stimulation without pituitary enlargement and (2) combinations of estrogens can bring about an inhibition of the usual pituitary enlargement without a corresponding inhibition of adrenal stimulation suggest that the estrogen-induced hyper-production of ACTH does not depend upon pituitary hypertrophy.

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Serum Lactic Dehydrogenase Levels in Various Disease States.* (22353)

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The elevation of serum lactic dehydrogenase (LDH) activity has been observed in association with a variety of diseases. Such elevations have been reported in humans with certain neoplastic diseases(1,2) and in mice with spontaneous, induced and transplanted tumors(3,4). High LDH activity has also been observed in patients with several non-neoplastic disease states, such as diabetic acidosis, myocardial infarction and hepatitis(2), although the number of cases is small and comparative levels of activity between various non-neoplastic and neoplastic diseases have not been specified. It is evident from such preliminary observations that a broad survey of a large variety of disease states is required in order to evaluate properly the clinical significance of changes in serum LDH levels and the usefulness of such determinations as a diagnostic tool. Furthermore, such a survey might suggest possible mechanism for alterations in serum LDH levels. In this report, then, we are presenting the results of such a survey, consisting of 130 individuals free of evidence of clinical disease, and 338 unselected hospital admissions.

Material and method. Clotted venous samples from human subjects were used for this investigation. The blood was allowed to stand at room temperature for no longer than 30 minutes and then centrifuged to separate serum from blood cells. For LDH determination serum samples thus collected were diluted prior to analysis. The micromethod of Strominger and Lowry(5) was used for the determination of level of enzyme activity and the results were recorded as millimoles of substrate oxidized per liter of serum per hour. Storing of uncentrifuged blood samples at 4°C not over one day gave the same result as that obtained with samples kept at room temperature for 30 minutes or less. However, clotted blood samples which remained at room temperature for as long as 60 minutes showed an increase in LDH activity by approximately 25% over those stored at 4°C or at room temperature for no more than 30 minutes. Heparinized and oxalated blood samples also gave LDH values about 40 and 60% higher, respectively, than refrigerated or 30 minute samples. The latter were therefore excluded from the data herein presented. The data have been grouped by organ systems, with subclassification into disease groups, and, except for a graphic presentation of findings in myocardial infarction, are all recorded in Table I. Multiple determinations were made in many cases, but except for those with myocardial infarction, an average was taken in each instance.

Results. (a) Normal controls. LDH was present in all human sera tested. Levels of serum LDH activity were determined in 68 normal females and 62 normal males ranging in age from 10 to 89 years. There was no significant variation with age or sex. The average for all 130 subjects was 15.1 ± 3.01, with a range of variation between 7.5 and 22.7.

(b) Cardiovascular system. There were 87 subjects with various cardiovascular diseases in this survey, and 40 of these were individuals with acute myocardial infarctions. The number of patients whose sera were tested at each time-period following onset of clinical symptoms is recorded at the appropriate points in Fig. 1. In all instances there was a confirming electrocardiographic diagnosis. Some patients were studied for as long as one month after onset of symptoms. The highest serum LDH levels occurred during the first 3 days, with values frequently 3-4 times normal; following this there was a gradual diminution to normal, which was usually observed between the 5th and 6th days after onset. In some instances there was a secondary rise between the 15th and 20th days, which was interpreted as possibly a secondary

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TABLE I. Serum Lactic Dehydrogenase Levels in Normal and Disease States.

Group	No. of cases	Avg	Range of variation
(a) Normal			
Females	68	14.6	7.6– 20.3
Males	62	15.7	9.1– 22.7
(b) Cardiovascular			
Myocardial infarct.*	40		
Hypertensive cardiovascular disease	38	17.5	3.8– 30.8
Mitral stenosis	3	18.0	16.7– 18.8
Subacute bacterial endocarditis	1	27.8	
Pulmonary infarct.	1	22.2	
Gangrene of lower extremity	4	17.7	10.6– 23.0
(c) Central nervous			
Cerebrovascular accident†	18	17.5	8.1– 31.0
Brain tumor	2	18.9	17.8– 19.9
Psychoneurosis	6	14.1	8.4– 16.6
(d) Gastro-intestinal			
Acute infection	13	16.1	10.5– 25.0
Gastric & duodenal ulcer	13	17.4	10.4– 25.9
Carcinoma, pretherapy	8	17.7	13.2– 25.7
post-therapy‡	12	17.1	12.6– 25.3
(e) Urinary			
Acute infection	17	17.0	11.5– 24.0
Chronic nephritis	6	20.8	12.7– 27.5
Carcinoma of kidney	3	18.4	12.5– 22.1
(f) Respiratory			
Acute infection—Lung	10	18.1	10.1– 26.2
—ENT	3	15.1	9.1– 24.3
Chronic " —Lung	3	21.1	17.2– 25.7
—ENT	3	15.9	11.0– 19.9
Carcinoma of lung			
Pretherapy	3	16.1	13.4– 21.4
Post-therapy	4	18.1	13.6– 22.1
Carcinoma of larynx			
Pretherapy	5	20.3	19.8– 21.8
Post-therapy	2	17.2	15.6– 18.7
(g) ♀ genital			
Non-malignant disease	8	13.7	4.8– 18.6
Carcinoma of cervix	4	14.6	9.7– 17.6
(h) Hemopoietic			
Infectious mononucleosis	5	27.2	23.6– 30.2
Thrombocytopenia, post-splenectomy	1	37.1	
Granulocytopenia§	2	14.3	9.4– 17.6
Leukemia, stem cell and myelogenous	4	38.9	24.3– 61.5
Leukemia, chronic lymphocytic	1	13.1	
Lymphoma, pretherapy	3	23.7	21.4– 27.4
post-therapy	3	24.1	13.9– 34.8
(i) Liver, pancreas and biliary tract			
Acute cholecystitis	2	17.1	13.4– 20.7
" infectious hepatitis	1	34.2	
" cholangitis‖	2	35.2	30.2– 40.5
Thorazine hepatitis	1	103.8	
Cirrhosis of liver	7	17.8	14.7– 23.6
Chronic cholecystitis	3	20.2	12.5– 24.4
Carcinoma—metastatic to liver	4	43.2	17.6–106.8
Carcinoma, gallbladder¶	1	40.2	
Ampulla Vater‖	1	36.1	
pancreas**	4	22.8	13.9– 39.4
(j) Breast			
Benign disease	4	15.0	11.3– 18.7
Carcinoma, pretherapy	4	13.8	3.5– 23.3
post-therapy††	8	30.1	5.9–106.8
(k) Miscellaneous			
Rheumatoid arthritis	4	25.1	20.5– 27.8
Fractures	3	15.6	12.0– 19.8
Osteomyelitis	1	19.0	
Diabetes	10	15.4	7.6– 21.4
Myxedema	2	19.7	17.2– 22.2
Hyperthyroidism	1	20.6	
Minor surgery	26	15.6	6.2– 21.4

* See Fig. 1.

† Approximately 40% showed 21.4 to 31.0.

‡ 4 cases had metastatic disease.

§ One case post-splenectomy.

‖ These cases all had obstructive jaundice.

¶ No obstructive jaundice; no metastasis to liver.

** Only 1 case had liver involvement and obstructive jaundice.

†† 5 cases had levels >25, and only 1 had known massive involvement of liver.

attack, but electrocardiographs failed to substantiate such an interpretation. (In addition, one case each of pulmonary infarction and subacute bacterial endocarditis showed slight elevations in serum LDH, but these were possibly associated with small foci of myocardial necrosis.) In 38 cases of hypertensive cardiovascular disease and in 3 with rheumatic heart disease and mitral stenosis values were within normal limits. There were a few cases in the former group with values between 25 and 30, and these may have been associated with small subclinical myocardial infarcts. Surprisingly, 4 subjects with arterial thrombosis and gangrene of the lower extremity also showed LDH values within normal limits.

(c) Nervous system. Neither cerebral infarct, hemorrhage nor primary brain tumor showed average levels of LDH significantly deviating from normal. However, in about 40% of individuals with cerebral infarction and/or hemorrhage LDH levels ranged between 21.4 and 30.

(d) Gastrointestinal tract. It is also evident from the data in this category that acute infectious processes (appendicitis and di-

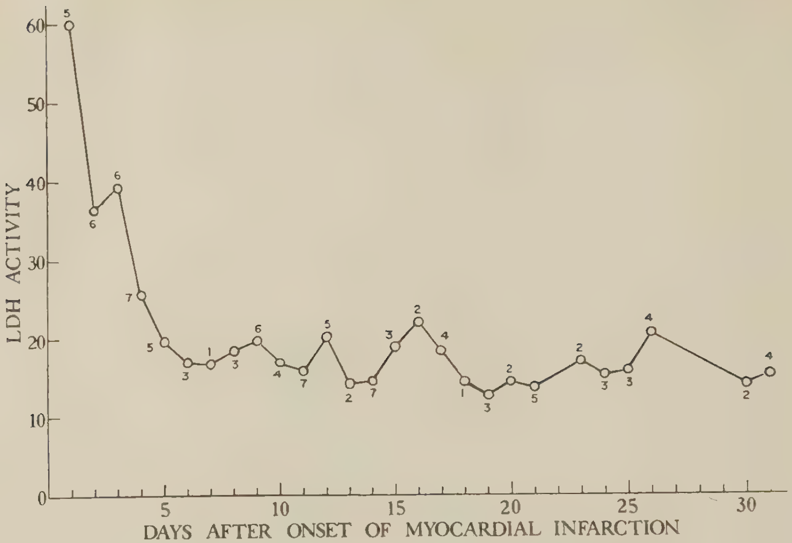

FIG. 1. Serum LDH activity in myocardial infarction.

verticulosis), gastric and duodenal ulcers, some of which were actively bleeding, and carcinoma (stomach, colon and rectum) are not associated with an increase in serum LDH activity. Furthermore, with regard to carcinoma there was no significant difference in LDH activity between patients with the primary tumor, some of whom showed widespread metastases, and those in whom the lesion had been recently resected and in whom no evidence of residual disease was present.

(e) Urinary tract. In 17 patients with acute renal and bladder infections, for the most part secondary to hypertrophy of the prostate, average LDH values were within normal limits, and this was also true of 6 chronic nephritics. There were 3 cases in the latter group with values in excess of 20, but these patients did not show elevated blood urea nitrogen levels, and in several other patients with an elevated blood urea nitrogen LDH values were also within normal limits. Urinary retention and uremia are therefore apparently not associated with elevated serum enzyme levels. In 3 instances of carcinoma of the kidney LDH values were not significantly elevated.

(f) Respiratory system. The results in patients in this group were essentially similar to those with diseases of the gastrointestinal tract. In cases with acute inflammatory disease these were for the most part instances of bronchopneumonia and acute sinusitis, and in the chronic inflammatory group chronic bronchitis and chronic tonsillitis. As regards carcinoma, there was again no significant difference between the pretherapy and posttherapy groups, the latter including patients who had undergone definitive surgery or who were receiving radiation therapy for metastatic disease.

(g) Female genital tract. In this category were included non-malignant diseases of the ovaries, tubes, uterus or cervix (benign tumors and chronic inflammation). as well as 4 cases of epidermoid carcinoma of the cervix. All showed levels of serum LDH activity within the normal range.

(h) Hemopoietic system. In the data shown under the hemopoietic system, infectious mononucleosis consistently gave moderately elevated LDH levels. LDH levels were most strikingly elevated in 4 patients, 2 with stem cell and 2 with myelogenous leukemia, but one patient with chronic lymphatic leukemia showed a normal LDH level. Patients with malignant lymphoma, including Hodgkin's Disease, showed levels comparable to that seen in infectious mononucleosis, with the exception of one case receiving x-ray therapy in whom LDH activity was within normal limits on several determinations. One patient with splenectomy for thrombocytopenia purpura showed a level about twice average normal; that this was probably not due to splenectomy as such is indicated by the fact that a post-splenectomy patient with granulocytopenia failed to show an increase in LDH activity.

(i) Liver, gallbladder, pancreas and biliary tract. As regards the 25 patients in this category it is evident that cirrhosis of the liver and inflammatory disease of the gallbladder did not produce significant change in LDH levels. However, with lesions that produce obstructive jaundice, such as common duct calculi, Thorazine and malignant tumors, as well as acute infectious hepatitis, LDH levels were elevated to about twice the normal average. Markedly elevated levels were observed in a patient with thorazine hepatitis and one with massive infarction of the liver associated with metastatic carcinoma from the breast.

(j) Breast. In the preoperative state patients with benign lesions and with malignant lesions of the breast without clinical evidence of metastasis showed no significant elevation in serum LDH. However, in 5 of the 8 cases with metastatic disease there was a significant elevation in enzyme activity, and in only one was there massive liver involvement as indicated above.

(k) Miscellaneous. In this category, only the group of 4 arthritic patients gave results deviating from normal. While these were only moderately elevated, they were, however, consistently over 20. One of these was a patient with disseminated lupus.

Discussion. Of the neoplastic diseases investigated, only stem cell and granulocytic leukemia, lymphoma and those involving hepatic metastasis were associated with a consistent elevation of serum LDH activity.

Of the non-neoplastic diseases studied, myocardial infarction, infectious mononucleosis, thrombocytopenia, hepatitis, and obstructive jaundice whether intra or extrahepatic, also gave similar increases in LDH. Since lymphoid, hepatic and cardiac tissues are relatively rich sources of LDH(6), it has been commonly suggested that if necrosis occurs, the enzyme might be released from the damaged tissue into the blood stream, therefore increasing serum LDH. If the tissues involved in disease processes have a low LDH content, one would not expect any significant increase in the total LDH activity. Therefore, they should fall somewhat in the normal range. Furthermore, some neoplastic transformation may depend upon other organs involved as metastatic sites; here, bone appears to be a common metastatic site, since most of these cases showed x-ray evidence of osseous metastasis. It remains for future studies to determine whether tumors of bone, primary or metastatic, are associated with elevated LDH levels.

In the cases of leukemia and infectious mononucleosis and thrombocytopenia the release of the enzyme might be the result of two different causes. First, it could be due to a change in cellular permeability, which possibly results in the secretion of the enzyme from the cells. Secondly, since white blood cells are relatively more fragile in the case of leukemia than normal white cells, the chance of break-down of white cells must be greater and the end result would therefore be the release of enzyme into the blood stream. Other tissues involving neoplasm would be disintegrated more frequently than the normal original tissues, therefore a high normal range level of serum LDH activity was observed in these patients. Other cases with acute infection or leucocytosis of moderate or severe degree showed slight elevation of serum LDH. This elevation might be related to the causes similar to those postulated previously.

The fact that carcinoma of intestine, stomach and kidney, 3 relatively rich sources of LDH(6), gave normal level serum LDH seems to reject the suggestion that serum LDH elevation was due to the release of the enzyme from tissues. While this concept is only plausible, it could be explained as due to some inactivation processes. The enzyme in the gastrointestinal and urinary tracts might be rendered inactive before it entered the blood stream. Furthermore, studies on the rate of urinary excretion of LDH in various neoplastic and non-neoplastic diseases may also prove fruitful.

Skeletal muscle and brain tissue of mouse were also pointed out as comparatively rich sources of LDH(6); however, there is no comparable study in human beings. If this finding could be applied to man, then gangrene of the lower extremity should give more striking results than myocardial infarction, and cerebro-vascular accidents should give data comparable to the latter. However, a normal level of serum LDH was noted. This might be explained as follows: The enzyme released from the necrotic tissues in the myocardium have ready access to the flowing blood stream in the cardiac chambers, thus resulting in an elevated serum LDH. Whereas in the leg and brain when occlusion of the major channel of blood occurs, the released enzyme does not have ready access to a blood supply, so the enzyme is destroyed or inactivated before reaching the circulatory system.

Our present studies did not confirm the elevation of serum LDH levels in diabetic acidosis as reported by Wróblewski and LaDue (2). Two of the 10 diabetic patients in the present investigation were in acidosis and the highest value obtained was 21.4. This might be due to the difference of degree in diabetic acidosis.

The mechanism by which serum LDH is increased in association with certain diseases is yet unknown. Experiments relating to the etiology may lead to a clearer understanding of this phenomenon. At present serum LDH determination may be used as a supplementary tool for diagnosing certain neoplastic diseases. It is a good index for myocardial infarction and diseases involving hepatic change.

Summary. The serum lactic dehydrogenase activity of normal human subjects and of patients with various diseases has been studied. Elevated levels were found in such neoplasms as stem cell and granulocytic leukemias, lymphomas, carcinoma of the pancreas and gallbladder, metastasis from carcinoma of the breast, and in metastatic disease of the liver. Increased enzyme activity was also noted in such non-neoplastic diseases as myocardial infarction, infectious mononucleosis, thrombocytopenia, acute infectious hepatitis and obstructive jaundice. Possible mechanisms of such elevation in serum LDH level have been discussed.

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Methemoglobinemia Induced by X-Irradiation.* (22354)

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As a first approximation it may be assumed that X-irradiation produces ion-pairs in aqueous solution as in air and for the same dose that the number of ion-pairs produced per unit weight is the same in solution as in air. A one-roentgen dose of X rays, by definition, produces 2.08 x 10^9 ion-pairs per 0.001293 g of air and may be considered to produce 1.6 x 10^{12} ion-pairs per ml of aqueous solution. Also, in aqueous solution containing oxygen there is reason to assume production of oxidant equivalent to 4 univalent atoms per ion-pair. Thus, one roentgen produces the equivalent of 6.4 x 10^{12} univalent atoms of oxi-

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* Supported by U. S. Atomic Energy Commission.

dant per ml of solution. Since one microequivalent corresponds to approximately 6 x 10^{17} univalent atoms, a one-roentgen dose of X rays will produce the order of 10^{-5} microequivalents of oxidant per ml of solution. This estimate of the oxidizing ability of X rays is based on the simplest assumptions about mechanism and probably is somewhat low(1); however, the observed degree of oxidation of most biological materials is less than expected from the estimate(2). A recent report(3) indicates that 25,000 roentgens of X rays applied to lysed human erythrocytes diluted 1:40 with phosphate buffer converted about 60% of the hemoglobin to methemoglobin. For present purposes erythrocytes may be considered to contain 20 microequivalents of ferrous iron in the form of hemoglobin. The solution of hemoglobin irradiated contained, therefore, 0.5 microequivalents of hemoglobin iron per ml. The dose of irradiation used would provide 0.25 microequivalents of oxidant per ml and might, therefore, convert 50% of the hemoglobin to methemoglobin. The conversion reported, 60%, although large compared to results obtained with other biological material(2), is about the same as might be expected from the oxidizing potential of the radiation. The report(3) also indicates that irradiation of the rat *in vivo* with 3000 roentgens led to the conversion of about 2% of the hemoglobin to methemoglobin. This represents oxidation of 0.4 microequivalents of hemoglobin per ml of erythrocytes. A second dose of 3000 roentgens given immediately after the first led to the oxidation of additional hemoglobin. The amount oxidized by the second dose was about twice that obtained with the first dose. 3000 roentgens would provide 0.03 microequivalents of oxidant per ml of erythrocytes and might, therefore, convert 0.15% of the hemoglobin to methemoglobin. Thus, the amount of hemoglobin found oxidized was 13-26 times as much as expected.

It is possible that practically all of the oxidizing potential of the radiation arising throughout the animal appeared in the erythrocytes by way of the circulation. Assuming the animals had a total volume of 300 ml, a red-cell volume of 15 ml, and all of the oxidant arising from 3000 roentgens eventually appeared in the red cells, each ml of the cells would be subjected to 0.6 microequivalent of oxidant. This amount is of the same order as the amounts of hemoglobin found oxidized by 3000 roentgens. An alternative explanation is that the effect of the irradiation *in vivo* was multiplied in some fashion. The relatively small chemical potential of a dose of radiation that will profoundly affect an animal, *e.g.* 1000 roentgens, has given rise to speculation about the possibility of a multiplication factor, *e.g.* chain reactions. No biological system considered *in vitro* has shown the degree of multiplication implied by the experiments under consideration(2).

Experimental. The preceding considerations appear in order although in the present work using another strain of rat given up to 5000 roentgens of whole-body X-irradiation (250 KVP, 0.5-2.8 mm Cu HVL) essentially no methemoglobin was detected in the blood. Test for methemoglobin was made as follows: Immediately after irradiation the animal was anesthetized with ether. Blood was taken from the heart in heparin. An equal volume of cold water was added. The mixture was kept in the cold for about 15 minutes. An equal volume of cold saturated ammonium sulfate, adjusted to pH 6.6 with fixed alkali, was added. After standing 15-20 minutes in the cold, the mixture was filtered. The clear filtrate was assayed spectrophotometrically for methemoglobin(4). In addition, the filtrate was tested for change in optical density at 630 mμ following addition of cyanide(5) and was examined with a hand spectroscope for the characteristic absorption band of methemoglobin.

In both irradiated and control rats the level of methemoglobin did not exceed 0.2% and no difference between the groups was observed.

Summary. Methemoglobinemia was not observed in rats given up to 5000 roentgens of whole-body X-irradiation *in vivo*.

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Influence of Vit. E Deficiency in Rabbit on Urinary Excretion of Free Amino Acids.* (22355)

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It has been shown that urinary excretion of free amino acids is elevated in patients with progressive muscular dystrophy(1). Also it has been reported that vit. E-deficient rabbits excrete extra quantities of free amino acids in urine although no actual data have been presented(2,3). In view of these considerations, and since any information concerning metabolic disturbances in vit. E deficiency may aid in elucidation of the biochemical role of the vitamin, we have determined the influence of vit. E deficiency on urinary excretion of amino acids by rabbits.

Methods. White New Zealand rabbits, weighing initially about 500 g each, were given the same purified diet previously described(4) except that folic acid was omitted. Controls received this diet with oral supplements of 10 mg of alpha tocopherol acetate 2 times per week. There were 4 rabbits in each group. The animals were kept in individual metabolism cages and daily urine samples were collected throughout the experiment. Urine samples were analyzed for free amino acids by the copper method(5). The experiment was terminated when unsupplemented animals exhibited early stages of muscular dystrophy.

Results. Vit. E deficiency led to a considerable increase in excretion of free amino acids in urine. At the time of appearance of earliest physical signs of muscular dystrophy urinary excretion of free amino acids was tripled (Table I). Actually there was a significant increase in excretion of amino acids before any physical signs of dystrophy were apparent. This agrees with the observation of Ames (2).

TABLE I. Influence of Vit. E on Average Daily Urinary Excretion of Free Amino Acids by Rabbits.

	mg amino N/kilo body wt	
Days on diet	Without vit. E	With vit. E
1- 5	6.6	5.2
6-11	5.9	4.5
11-15	4.5	4.3
16-20	5.7	3.7
21-25	8.6	3.3
26-31	13.5	4.5

Increased excretion of free amino acids in vit. E deficiency may indicate an impaired incorporation of amino acids into protein, an increased breakdown of tissue proteins, or an impairment in normal metabolic disposal of amino acids. Experiments with C^{14} labeled glycine have indicated that vit. E deficiency does not significantly affect incorporation of that amino acid into protein or its oxidation to CO_2(6). This would suggest that elevated excretion of free amino acids in vit. E deficiency is due to accelerated breakdown of tissue protein, probably primarily skeletal muscle.

Summary. Vit. E deficiency in the rabbit results in increased excretion of free amino acids in urine. This increase in amino acid excretion is apparent before any physical signs of dystrophy are observed.

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Sedimentation Behavior of Desoxypentose Nucleic Acids from Normal and Pathologic Human Spleens. (22356)

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Previous work in this laboratory has demonstrated that the desoxynucleic acids (DNA) of normal and leukemic human cells differ in some physico-chemical characteristics(1,2). Interest in this study of macromolecular structures of normal and malignant cells has been greatly stimulated by the "template hypothesis"(3-5). According to this hypothesis, neoplastic growth would be the consequence of an abnormal event in the self duplication of some macromolecules in the cell(6), resulting in a malignant transformation of some cytoplasmic and/or nuclear structures.

Although the "template hypothesis" concerns primarily the role of ribonucleic acid (RNA) in the mechanism of specific protein synthesis, it could be extended to the role of DNA in mitosis and gene duplication. For this reason the sedimentation constant of DNA from normal and leukemic cells was studied.

Material and methods. DNA was prepared from calf thymus, normal human spleen removed surgically after traumatic rupture, human spleens from cases of multiple myeloma, Brill-Simmers disease, myeloid leukemia and hereditary spherocytosis. The organs were frozen with dry ice immediately after removal and powdered in a high speed mixer with additional dry ice. Thirty grams of powdered tissue were suspended in 100 ml of NaCl-Na Citrate solution (0.14 M NaCl; 0.01 M Na Citrate) and homogenized for 2 minutes at 12,000 r.p.m. The material was then filtered through 3 layers of gauze, centrifuged, and the sediment washed 3 times. The sediment was then resuspended in 20 volumes of 1.7 M NaCl-0.01 M Na Citrate solution and homogenized again for 30 seconds at a lower speed. DNA sodium salt was extracted according to the method of Gulland (7) as modified by Chargaff *et al.*(8), then dialyzed against distilled water for 72 hours at 0-4°C and lyophilized. The N/P ratio as well as other chemical properties of the obtained DNA, correspond closely to the theoretical values and have been published elsewhere(2). DNA was dissolved in cacodylate buffer of ionic strength 0.3 and pH 7(9) by gentle overnight stirring in the cold room (0-4°C). The DNA concentration of the solution was calculated from its phosphorus content (P DNA) determined with the method of Fiske and Subbarow, after conversion of the arsenic from the penta- to the trivalent form(10). The samples were analyzed at room temperature within 24 hours of being dissolved. Sedimentation data were obtained in the Spinco Model E analytical ultracentrifuge. Sedimentation constants were corrected for a solvent having viscosity and density of water at 20°C; the partial specific volume of DNA was assumed to be 0.55(11,12).

Results. Typical schlieren photographs of DNA sedimentation boundaries are seen in Fig. 1. The data obtained with DNA from a series of normal and pathological spleens are shown in Fig. 2 where the S_{w20} values are plotted against the P DNA concentration of the samples. It can be seen that points fall on the same curve, regardless of the source of the DNA samples.

Discussion. The spread of values obtained with any given P DNA concentration seems to indicate a variability within each determination rather than differences in the sedimentation constant of DNA samples of dif-

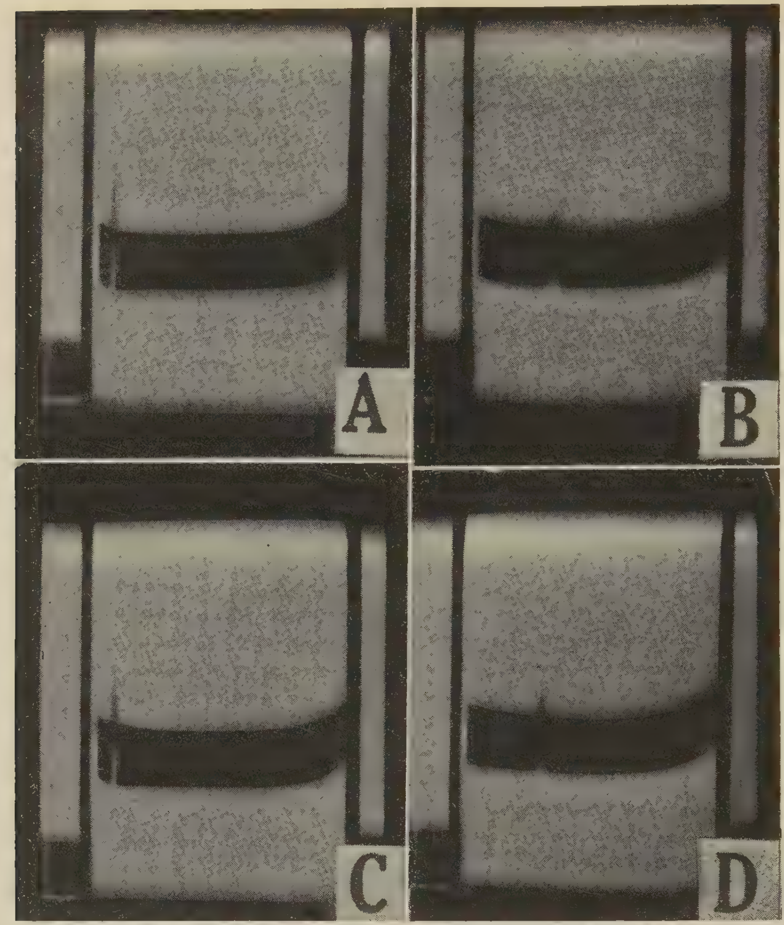

FIG. 1. A and B. Schlieren photographs of sedimenting boundaries of DNA from spleens of patients with multiple myeloma. Speed, 59,780 rpm. Bar angle, 45°. Time, 16 min. Concentration, 1.7 mg P DNA/100 ml. C and D. Schlieren photographs of sedimenting boundaries of DNA from spleens of patients with myeloid leukemia. Speed, 59,780 rpm. Bar angle, 45°. Time, 16 min. Concentration, 1.9 mg P DNA/100 ml.

ferent origin. These results do not support the contention that DNA from normal and pathological human cells have different sedimentation constants. Extrapolation of the data to concentration zero indicates an S_{w20} value of about 20 to 21, in agreement with the data of Peacocke and Schachman(9) and of Doty(12). Other authors(12-16) have reported lower S_{w20} values at zero concentration although at a concentration of DNA of 9-18 mg %, the sedimentation coefficients reported by them are in agreement with ours. This difference in the extrapolated values may be due to the different concentration range of the samples and to the different ionic strength of the buffer solutions used by various investigators. Sadron(17) and Jordan (18) have shown that some physical properties of DNA, such as viscosity, are strongly influenced by the ionic concentration of the solution, and that reproducible data can be obtained only when all free negative charges of DNA are saturated. Furthermore Peacocke and Schachman(9) have demonstrated that, when DNA is sedimented in distilled water at a concentration below 60 mg %, it behaves like degraded DNA and that the sedimentation constant increases up to an ionic strength of 0.3. Further addition of electrolytes has no greater effect.

Very strong intermolecular forces act between DNA molecules at relatively high con-

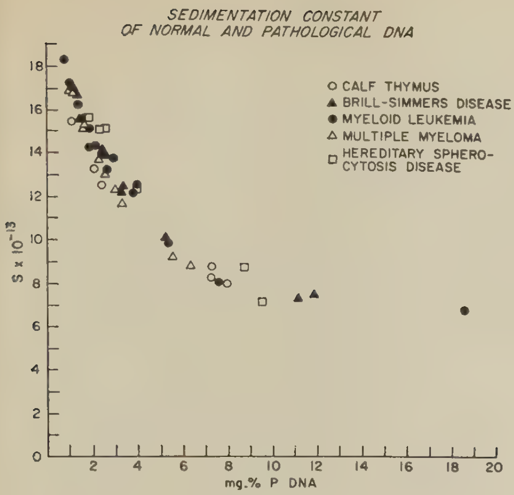

FIG. 2.

centrations, and may disturb the sedimentation rate even at the lowest concentrations used in this work. This may make the demonstration of heterogeneity in various DNA samples difficult. While this suggests the need for further studies of the sedimentation behavior of DNA at low concentrations with more sensitive recording systems (such as the optical absorption method used by Butler and Shooter(19)), the results reported in this paper indicate that DNA samples from normal and tumoral cells give identical sedimentation curves over the large range of concentrations studied.

Summary. 1. Sedimentation curves of DNA from spleens of normal individuals and patients with diseases causing a leukocyte infiltration in the spleen were obtained by means of the ultracentrifuge. 2. DNA was dissolved in cacodylate buffer (pH 7; u 0.3) at concentrations range 1-20 mg % of P DNA. No differences in sedimentation curves of DNA from normal and infiltrated spleens were noted.

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Comparative Study of Vitamin B_{12} Assay in Urine. (22357)

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In a study of cyanocobalamin (vit. B_{12}) excretion in the urine of patients, following an intramuscular (I.M.) dose of 50 μg of crystalline B_{12}, striking variations in the apparent content of B_{12} were observed(1,2). These variations stemmed from differences in the specificity of the assay organisms. It is known that some of the microorganisms serving for the microbiological assay of B_{12} respond to congeners of the vitamin and also deoxyribonucleosides(3,4). Specificity, better than that of bacteria, is shown by *Euglena gracilis* and, above all, by *Ochromonas malhamensis* (5,6). Here we compare the response of *Lactobacillus leichmannii, E. gracilis* and *O. malhamensis* to the B_{12} family of vitamins as they occur in urine.

Methods. The maintenance media for the 3 organisms have been given(7-9). The assay technics used have been outlined in previous publications(10). *L. leichmannii* grew in 24 hours, the protozoans within 4-6 days. The inoculum for the bacillus was prepared by 1:10 dilution with basal medium of a 24 hour culture. The inoculum for *E. gracilis* was prepared by 1:50 dilution of a 4-6 day culture in the basal medium. The *Z* strain rather than the *bacillaris* strain of *Euglena* was employed, as it attained higher densities in a shorter period and had the same sensitivity and specificity(11,12). *O. malhamensis* inoculation was directly from a 4-6 day culture in the maintenance medium without significant carry-over of B_{12} activity. The assay media for the organisms were given(6, 8,13). Urines from control patients, who had not received I.M. B_{12}, were diluted 10 X with buffer at pH 4.5,[†] and autoclaved for 30 minutes to convert bound cobalamin into its microbiologically active form. This procedure allows estimation of total B_{12}(8). All urines were diluted, as necessary, for the measurements to fall on the straight-line portion of the standard curve. Urines from control patients were further diluted 1:2 - 1:100 with assay medium (final dilution of the urine 1:20 - 1:100). Urines from patients, who received 50 μg of B_{12} I.M., were diluted 50 X, autoclaved, and subsequently diluted again 1:2 - 1:100 (final dilution 1:100-1:5000). *L. leichmannii* was incubated at 37° for 24 hours; the protozoans were kept at room temperature (not exceeding 30°C) for 4-6 days under constant illumination provided by six 40 W. warm white fluorescent tubes at an average distance of 0.8 m. Growth was measured as optical density units with a Welsh Densichron equipped with a red-sensitive probe.

Results. Recovery of added vit. B_{12}: To test the validity of the assay methods, recovery experiments of B_{12} in urine were done with 4 concentrations of B_{12} (Table I). The 2 protozoans yielded satisfactory recoveries from urine. In recovery experiments with sera and cerebrospinal fluids, we noticed that *O. malhamensis* may be slightly stimulated by other materials in cerebrospinal fluid and serum when compared to *Euglena*. These stimulants did not affect the results in urine.

B_{12} in Urine. Table II shows results with urine collected during the first 8 hours after intramuscular injection of 50 μg of vit. B_{12} in 23 individuals. In all except 2 urines (Nos. 1 and 5) values obtained with *O. malhamensis* were lower than for that with *Euglena*. The values with *L. leichmannii* were higher than that with *Euglena* except in 2 cases (Nos. 18

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[*] This work was aided by grants from the U. S. Public Health Service, Division of Nutrition and Metabolism, the National Multiple Sclerosis Society, and from Mrs. Oliver Smith, New York City.

[†] One-half gram of trans-aconitic acid/100 ml of distilled water, to which triethanolamine had been added to reach pH 4.5. One hundred mg of Na metabisulfite had also been added to 100 ml of buffer for stabilization of the analogs of B_{12} e.g. hydroxycobalamin(14,15).

TABLE I. Recovery of B_{12} Added to Normal Undiluted Urine.

Conc. (μg/ml)	*L. leichmannii*	*E. gracilis*	*O. malhamensis*
5	10.8	3.6	3.7
10	16.5	8.4	9.5
15	24.1	14.2	14.2
20	31.0	18.2	18.2

and 19). In no instance were values with *L. leichmannii* lower than with *O. malhamensis.*

Discussion. Ford(6) has evaluated the response of the 3 microorganisms to pseudovitamin B_{12} and to other B_{12}-like factors. This evaluation is used here as the basis for calculation of true B_{12} values as compared with values for pseudo-B_{12}'s. Ford and Hutner also have discussed the positive response of *L. leichmannii* to deoxyribosides(4). The main difference of practical interest between *Euglena* and *Ochromonas* is the high response of the former to pseudo-vitamin B_{12}. With *L. leichmannii* the response to pseudo-B_{12} is half that of *Euglena.* We assume that the response to *O. malhamensis* (OM) gives the true value for vit. B_{12}. Therefore: $B_{12} = OM$. The extra response of *Euglena* (E) as compared with *Ochromonas* is assumed to reflect the pseudo-B_{12}'s (Ps_{12}) in the sample. Pseudo-B_{12}'s may be estimated by the formula $Ps_{12} = \frac{E - OM}{8}$.

Besides responding to certain pseudo-B_{12}'s, *L. leichmannii* (L) also responds to thymidine and certain other deoxyribosides(3). The quantity of these deoxyribosides (D) may be computed in terms of true vit. B_{12} by the following calculation

$$D = L - OM - 4\frac{E - OM}{8}$$

which leads to $D = L - \frac{OM + E}{2}$.

These formulae are only crude approximations, as they disregard the possible presence of other members of the vit. B_{12} group. If by these calculations interference by deoxyribosides is suspected, it might be desirable to assay for them directly(16). Results in Table II indicate a correlation between pseudo vit. B_{12} and true B_{12}. Deoxyribonucleosides, and other factors, stimulating the growth of *L. leichmannii* appear to interfere in the vit. B_{12} load tests described here; the difference in results between *L. leichmannii* and the others are possibly due to dietary differences of patients, which may be reflected in their urine.

The recoveries of true B_{12}, according to Table I, appear to be 6-11 μg too high with *L. leichmannii.* This excess may perhaps be ascribed to the combination of stimulatory urinary constituents with materials present in the basal medium(17), a reaction, which assumes disturbing proportions, when the growth is dense.

Summary. The response of *Ochromonas malhamensis, Euglena gracilis,* and *Lactobacillus leichmannii* to vit. B_{12} in normal urine and in urine of patients, given an intramuscular dose of 50 μg of vit. B_{12}, was studied. The "true" vit. B_{12} values presumably are given by the *Ochromonas malhamensis* assay. The response of *E. gracilis* and *L. leichmannii* to other members of the B_{12} group and to other metabolites is discussed from the standpoint of how to estimate responses other than *O. malhamensis*-active B_{12}. Calculations are given for the estimation of interfering metabolites.

TABLE II. B_{12} in Urine of Patients within 8 Hours after Intramuscular Injection of 50 μg of B_{12}.

Total output in μg determined by response of		
Ochromonas	*Euglena*	*Lactobacillus*
.6	.5	1.2
1.0	2.4	3.0
1.0	2.9	10.6
1.5	1.7	5.0
1.9	.7	3.8
2.5	4.2	8.3
3.6	5.6	9.9
3.8	7.4	7.4
4.1	6.9	8.5
4.9	10.2	23.4
6.0	14.5	33.0
6.3	11.6	16.8
6.6	13.1	20.0
8.1	10.2	20.4
9.0	9.5	19.0
10.2	16.1	19.5
10.5	22.7	33.1
11.4	38.0	24.7
11.7	17.1	11.5
11.9	15.0	16.1
13.7	23.6	33.0
13.8	24.7	27.6
28.7	30.2	31.3

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Molybdenum Toxicity in the Rat.[*] (22358)

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The symptoms of molybdenum toxicity have been thoroughly described in several species(1), yet little is known of the underlying defects responsible for these symptoms, except that the resultant anemia can be remedied by copper sulfate supplementation. Weil-Malherbe and Green(2) and Lutwak and Sacks(3) have noted that compounds such as adenosine triphosphate (ATP) and acetyl phosphate are rapidly hydrolyzed in the presence of molybdenum salts. Since molybdate ions *in vitro* catalyze the acid hydrolysis of "high-energy" phosphate esters, the possibility existed that molybdenum might have a similar effect *in vivo*. If this were so, a decrease in the supply of "high-energy" phosphate compounds might then be fundamental to some of the symptoms of molybdenum toxicity. The acetylation of p-aminobenzoic acid (p-ABA) and the conjugation of glycine with benzoic acid have been used extensively in tests for liver function. Both reactions involve ATP, coenzyme A, magnesium ions, and the appropriate enzymes. Lipmann(4) has shown that ATP is involved in the acetylation of sulfonamides by pigeon liver preparations, and Chantrenne(5) has established a requirement for ATP in the enzymatic formation of hippuric acid. If molybdate ions *in vivo* should catalyze the hydrolysis of organic phosphate esters, then the supply of "high-energy" phosphate compounds might be reduced, and presumably, the reduction would be reflected in the ability of the animal to acetylate p-aminobenzoic acid or to conjugate benzoic acid.

The experiments reported here tested this hypothesis. Studies were also conducted on the effect of molybdenum toxicity on the alkaline phosphatase activity of various tissues.

Methods. The basal diet had the following percentage composition: casein, 20.0; glucose, 69.0; corn oil, 6.6; salt mixture, U.S.P. XIV, 4.0; choline chloride, 0.1; 2500 I.U. vit. A, 250 I.U. vit. D, 15 mg alpha-tocopherol in corn oil, 0.3. Other vitamins were provided in the following quantities, mg/100 g diet: thiamine hydrochloride, 0.5; riboflavin, 0.8; niacin, 4.0; pyridoxine hydrochloride, 0.5; calcium pantothenate, 4.0; biotin, 0.04; folic acid, 0.2; menadione, 0.5; vit. B_{12}, 0.003. The basal diet was supplemented with 4.0 mg copper/100 g in order to provide sufficient copper to prevent anemia. Albino rats of the Wistar strain were obtained from commercial sources and housed in individual cages. In the first experiment sodium molybdate was added to provide a dietary level of 0.08% molybdenum. The average initial weight of the 8 pairs of rats studied was 94 g. For each pair, the food intake of the control rat fed the basal diet was limited to the intake of the rat fed the molybdenum-supplemented diet. After one week on the experimental diets, the rats were placed in metabolism cages and injected intraperitoneally with a 2.5 mg dose of p-aminobenzoic acid. Urine was collected for 24 hours after injection. This procedure was carried out once a week for 4 successive weeks with each pair of rats. The animals were sacrificed at the end of 6 weeks. In the second experiment, the dietary levels of molybdenum were 0.08%, 0.12% and 0.14%. The average initial weight of the rats was 79 g. All diets were fed *ad libitum*. After 2, 3, and 4 weeks, rats from each group were injected with a 2.5 mg dose of p-aminobenzoic acid as described in Exp. 1. Total and free urinary p-aminobenzoic acid were measured by the method of Bratton and Marshall(6). Urinary hippuric acid was measured following injection of benzoic acid by the method of Alexander *et al.*(7). Alkaline phosphatase activity was determined by the method of Bessey *et al.*(8), in which the hydrolysis of p-nitrophenylphosphate to p-nitrophenol is measured. Activities are expressed as micromoles of phosphate released in 30 minutes per mg protein. Tissues were homogenized in appropriate amounts of ice water in a Ten Broeck homogenizer. The protein content of the homogenates was estimated by the method of Lowry *et al.*(9).

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TABLE I. Effects of Dietary Molybdenum in Pair-Fed Rats.

Group	Body wt (g)	Urinary acetyl-p-aminobenzoic acid (% of total p-ABA excreted)	Liver alkaline phosphatase (%)
Control	191	49.0 (28–72)	100
+Mo 800 p.p.m.	188	53.6 (37–80)	177

Eight pairs of rats used. Urinary p-ABA values represent means of 4 replicates at 1 wk intervals or 32 assays. Range of values is given in parentheses. Liver alkaline phosphatase values are means of 8 assays.

Results. Growth and food intake. In the first experiment (Table I), the addition of molybdenum to the diets at a level of 0.08% caused a negligible depression in growth in contrast to previous results from this laboratory when younger animals from a different commercial source were used(10). Since a dietary level of 0.08% molybdenum failed to produce a severe toxicity in the first experiment, 3 levels of molybdenum were used in Experiment 2 and, in addition, younger animals were used. In this experiment, molybdenum, at all 3 levels, definitely retarded growth. The average weights at 5 weeks were 171, 121, and 87 g, respectively, for the rats fed the 0.08%, 0.12%, and 0.14% levels whereas the average weight of the control group was 218 g (Table II). Many of the rats developed an intermittent diarrhea, but there was no mortality. Prior to start of the experiment, all the rats gained normally on the basal diet. However, within 2 days after the start of the experimental period, the presence of molybdenum in the diet depressed food intake, especially at the two higher levels, and the weight gains reflected the reduced food intake. This was not due to decreased "palatability" of the diet since 5 rats given sodium molybdate by stomach tube, also showed poor food intake.

Acetylation of p-aminobenzoic acid. The addition of 0.08% molybdenum to the diet in the first experiment did not alter the ability of the pair-fed rats to acetylate a 2.5 mg dose of p-aminobenzoic acid (Table I). Likewise,

TABLE II. Influence of Various Levels of Dietary Molybdenum on Rat Growth, Tissue Phosphatase, and Acetylation of p-ABA.

Groups	Body wt (g)	Tissue alkaline phosphatase Liver	Heart	Kidney	Urinary acetyl p-ABA (% total p-ABA excreted)
		μM phosphate/30′/mg protein			
Control	218	.041 ± .004	.224 ± .021	3.709 ± .102	67 (47–87)
+Mo 800 p.p.m.	171	.085 ± .008	.178 ± .013	3.671 ± .192	53 (46–60)
+Mo 1200 p.p.m.	121	.263 ± .025	.203 ± .027	2.395 ± .364	60 (44–83)
+Mo 1400 p.p.m.	87	.220 ± .016	.218 ± .006	2.477 ± .166	52 (42–68)

Urinary p-ABA values were obtained on 6 rats/group; values in parentheses give range of values.

higher levels of molybdenum had little effect on the acetylation (Table II). Under these conditions, then, the supply of "high-energy" phosphate esters, and presumably the amount of acetyl coenzyme A available for the acetylation of p-aminobenzoic acid, was not affected by the presence of molybdenum *in vivo*.

Conjugation of benzoic acid. Following the injection of benzoic acid, the hippuric acid was isolated from urine in a one part butanol, 5 parts chloroform mixture, hydrolyzed and the glycine determined as formaldehyde with chromotropic acid. A considerable variation in response was observed to the intraperitoneal injection of 24 mg of benzoic acid. Both control and molybdenum-toxic animals were inconsistent in the excretion of hippuric acid, however, there did not appear to be any tendency for the toxic animals to excrete less of the conjugate. In one experiment of 10 control and 10 toxic rats the mean excretion was 2.2 and 2.8 mg glycine of hippuric acid, respectively, in 24 hours following administration. This supports the observations on p-ABA acetylation that molybdenum does not interfere to any extent in the occurrence of these endothermic reactions. It seems reasonable that molybdenum does not have an effect because of the higher pH of the tissue systems or due to a conjugation of the mineral with proteins of the tissues.

Tissue alkaline phosphatase. While the gross effects of molybdenum were rather slight in the first experiment, in every case, liver alkaline phosphatase activity was greater in the molybdenum-fed animal than in the pair-fed control (Table I). Although the significance of the increase in enzyme activity is unknown, the activity of this enzyme does seem to be a sensitive indicator of dietary molybdenum, and may be of potential use in the detection of molybdenum toxicity.

In the second experiment (Table II) all 3 levels of molybdenum significantly increased the activity of the liver enzyme ($P<0.01$), and the activity was significantly greater in the rats fed the 0.12% and the 0.14% levels than in the rats fed the 0.08% level ($P<0.01$). Increasing the level of molybdenum from 0.12% to 0.14% produced no additional increase in liver alkaline phosphatase activity. Heart alkaline phosphatase was unaffected.

Upon autopsy, the kidneys of the rats fed the 0.12% and the 0.14% levels of molybdenum were noticeably paler than those of the control rats or the rats fed 0.08% molybdenum, although the kidneys of the latter animals frequently were paler than those of the controls. These differences appeared consistently although the gross structure of the kidney seemed normal. This alteration, together with the possible role of alkaline phosphatase in the reabsorption of glucose in the kidney tubule(12), suggested that a study of kidney alkaline phosphatase might be valuable. The data in Table II show the effect of molybdenum on kidney alkaline phosphatase. In the rats which had been fed the diets for 4-7 weeks, the 0.12% and 0.14% levels of molybdenum significantly ($P<0.02$) depressed the activity of the kidney enzyme.

Some studies were made of the influence of dietary molybdenum on intestinal alkaline phosphatase activity. It was observed that after one week on the experimental regimen 5 rats receiving 1200 p.p.m. molybdenum had significantly less ($P<0.01$) intestinal phosphatase activity than 5 controls. Control and toxic values were 16.58 ± 1.64 and 4.46 ± 0.73 μM phosphate released/30′/mg protein respectively. Because of this rapid and pronounced effect, the influence of sodium molybdate on the intestinal phosphatase assay system was tried. Molybdate concentrations up to 10^{-4} M had no influence on a semi-purified preparation of intestinal phosphatase. At 10^{-3} M molybdate, a 20% inhibition of activity was observed. This is in agreement with experiments on liver preparations(10) and suggests that the decrease in activity observed in toxicity is probably a reflection of altered synthesis of the enzyme rather than a direct influence of the mineral on the enzyme assay system.

Summary. 1. High levels of dietary molybdenum did not alter ability of rat to acetylate p-aminobenzoic acid or to conjugate benzoic acid with glycine. 2. A depression of food intake and growth was evident within 24 hours after addition of 0.12% molybdenum to the diet. 3. Liver alkaline phosphatase activity was significantly increased in molybdenum-toxic rats whereas the activities of the kidney and intestinal alkaline phosphatases were significantly reduced.

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Structure and Function of Mitochondria in Irreversible Hemorrhagic Shock, I.*† (22359)

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Irreversible hemorrhagic shock is seen in humans and experimental animals who have suffered a prolonged period of hemorrhagic hypotension and are refractory to blood volume replacement therapy. Battlefield experience with massive blood transfusions in World War II and the Korean conflict(1) strengthened the belief of some workers that an irreversible intracellular metabolic change was taking place. Several studies involving tissue analyses have appeared during the last decade and have been ably summarized by Engel(2). In general, high levels of glucose lactate, pyruvate, amino acids and inorganic phosphate were found in the blood while the tissue stores of glycogen, ATP[‡], and coenzymes appeared depleted. Edwards *et al.*(3) recently repeated some of the key measurements in dogs. Early enzymatic studies were conducted by Russell, Long and Wilhelmi(4, 5) Burdette(6) and Hannon and Cook(7) using liver and heart slices of rats subjected to hemorrhagic shock: in some of these experiments lowered oxygen uptakes were observed. Thus, both the analytical and the enzymatic data suggested failure at the mitochondrial level. Harman(8-11) described a series of degenerative changes occurring in mitochondria when isolated by differential centrifugation and exposed to various environmental conditions. These alterations in structure (swelling and "crescent" formation) were accompanied by functional changes *e.g.* low P/O ratios and variations in oxygen uptakes.

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† The authors wish to thank Dr. E. R. Schmidt and Dr. C. W. Crumpton for their continued encouragement and support; Dean C. A. Elvehjem and the Wisconsin Alumni Research Foundation who kindly helped finance the initial phases of the project; and Dr. P. P. Cohen for his interest and helpful advice.

The two lines of thought, *i.e.* the evidence suggesting mitochondrial involvement in the phenomenon of irreversibility in clinical hemorrhagic shock and the interesting correlation between mitochondrial structure and function observed in *in vitro* experiments raised the following questions: Are mitochondria morphologically and functionally altered in irreversible hemorrhagic shock and do such *in vivo* changes resemble the *in vitro* changes described?

Methods. Production of shock.[§] Healthy mongrel dogs maintained on a standard *ad libitum* diet of dried dog biscuits were paired as nearly as possible as to sex, age, breed and weight and anaesthetized with 30 mg/kg pentobarbital sodium. One animal served as a control while the other was subjected to hemorrhagic shock by a modification of the Wigger's technic(12). Blood was withdrawn rapidly so that an arterial pressure of 40 mm Hg was produced in approximately 5 minutes. Subsequently blood was withdrawn and reinfused as necessary to maintain the initial hypotensive level.[||] The animals were sacrificed after 3 to 5 hours by removing the left ventricular musculature surgically. The members of the pair died within 15 minutes of each other. *Formalin-fixed preparations.* 5 g of heart muscle cut into thin slices were transferred to a semi-micro Monel cup containing 1 g sodium acetate in 50 ml of cold 10% formaldehyde and were ground on a Waring blendor base at full speed for 45 seconds. Liver and kidney samples were prepared in the same manner but blendorized at half speed for 15 seconds. The homogenates were strained through cheese cloth and examined under oil immersion in a Spencer phase contrast microscope using Dark-M contrast.

Isolation of mitochondria. 15 g heart were minced and ground for 90 seconds in 75 ml semi-frozen 0.38 M sucrose containing 100 μM K_2HPO_4 using a semi-micro Monel cup in a standard Waring blendor base. Another 75 ml of the sucrose solution were stirred in and the suspension strained through cheese cloth. The preparations from the control and the shocked dogs were thus made within 15 minutes of each other and centrifuged simultaneously in a refrigerated international centrifuge. The mitochondria were sedimented between the speeds of 450 and 1300 x g, suspended in 0.5 M sucrose, dispersed by means of a glass homogenizer and the densities of the suspensions adjusted nephelometrically. The total nitrogen concentration was estimated by Johnson's method(13). The mitochondria were thus isolated in hypertonic sucrose because of Harman's findings(10,11) that under such conditions degenerative changes ("crescent" formation) were negligible, an observation which we have been able to confirm. *Enzymatic activities* were estimated by the conventional Warburg method at limiting levels of mitochondria under the conditions outlined in Fig. 4. In order to determine P/O ratios the incubation mixtures were fortified with 50 μM fructose and 2 mg

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[‡] The following abbreviations will be used: AMP = adenosine − 5′ − phosphate; ATP = adenosinetriphosphate; P/O ratios = micromoles of inorganic phosphate esterified per microatom of oxygen consumed.

[§] The experiments described were carried out between July and Nov. 1955.

[||] Baxter blood bottle with ACD solution 1.38% Na-citrate, 1.58% dextrose and 0.5% citric acid were used to collect the blood.

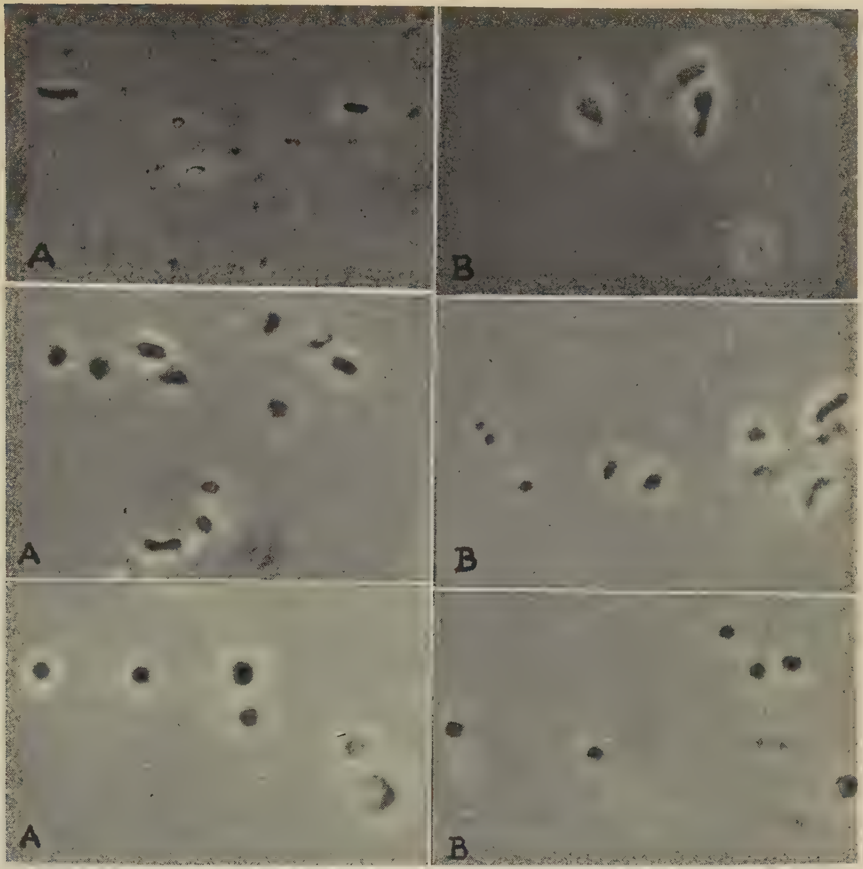

FIG. 1 (top). Heart mitochondria from normal and shocked dogs prepared in formaldehyde and examined under phase-contrast. a, normal; b, shock. Magnification app. 2300 ×.

FIG. 2 (middle). Heart mitochondria from normal and shocked dogs isolated by differential centrifugation, suspended in 0.5 M sucrose and examined under phase-contrast. a, normal; b, shock. Magnification app. 2300 ×.

FIG. 3 (bottom). Heart mitochondria from normal and shocked dogs aged in 0.5 M sucrose and examined under phase-contrast. a, normal; b, shock. Magnification app. 2300 ×.

yeast hexokinase (Sigma, type II) which were poured in from the side arm shortly before the taps were closed. Inorganic phosphate levels were determined by a modification of the Fiske-Subbarow method. In a few experiments fluoride was added as a phosphatase inhibitor but the results were not affected materially by its presence. In general the experimental mixtures were kept as simple as possible in an effort to maintain the mitochondria in as nearly their native state as feasible.

Results. Morphology of formalin-fixed preparations may be observed in Fig. 1. The grinding breaks up the long chains of mitochondria which accompany the strands of myofibrils in the undisturbed tissue. The free floating normal particles tend to be square to spindle-shaped rods with sharp boundaries and dense to moderately translucent centers. At times they may be slightly knobby. The particles encountered in preparations from shocked dog hearts are swollen and irregular in shape with indistinct boundaries and often have translucent centers. Blendorates from livers and kidneys also showed abnormal mitochondria. The swollen spheres and "crescents" which are associated with degenerative changes in Harman's *in vitro* experiments have not been observed in formalin-fixed preparations.

Structure of mitochondria isolated in su-

TABLE I. Oxygen and Phosphate Uptakes of Heart Mitochondria from Normal and Shocked Dogs in the Presence of the Hexokinase System.

	Normal			Shock			
Substrate	O_2 uptake μatoms /mg N*	P uptake μmoles /mg N*	P/O	O_2 uptake μatoms /mg N*	P uptake μmoles /mg N*	P/O	Hr of shock
α-Ketoglutarate	12.1	19.5	1.6	10.3	11.8	1.1	2
	9.9	16.9	1.7	10.4	20.7	2.0	3
	9.8	25.9	2.6	5.9	13.8	2.3	3½
	11.4	23.4	2.1	5.1	8.3	1.6	5
	9.8	22.7	2.3	6.8	10.5	1.5	5
	8.6	14.8	1.7	4.9	3.6	.7	5
	14.8	30.9	2.1	8.8	14.8	1.7	5½
Succinate	10.4	19.6	1.9	11.7	13.2	1.1	3
	14.3	31.3	2.2	13.5	20.6	1.5	3
	10.8	22.5	2.1	11.0	23.0	2.1	5

* Avg of duplicate or triplicate flasks.

Main compartment: 20 μM $MgCl_2$, 3 μM AMP, 50 μM phosphate buffer, pH 7.5, 40 μM substrate, mitochondria (app. 1 mg N) and 0.5 M sucrose to 1.8 ml; sidearm: 50 μM fructose and 2 mg hexokinase in 0.2 ml; center well: 0.2 ml 2 N NaOH; temperature: 30°C; gas phase: oxygen.

crose suspension is demonstrated in Fig. 2. Rods persist in varying numbers, often as high as 80%; the remaining mitochondria appear as spheres of varying sizes. "Crescents" are not seen in fresh suspensions. Preparations from shocked dogs generally have a lower rod count and show a preponderance of the small and medium sized spheres while the normal tends to contain large forms. This difference in size is often very marked and is observed also in the incubation mixtures at the end of Warburg experiments. A comparison of Fig. 1 and 2 reveals that the normal mitochondrion tends to be small in formalin but large in sucrose whereas the abnormal behaves in an opposite manner.

On aging at room temperature swollen spheres and "crescents" will be formed in both types of preparations. However, this *in vitro* change tends to occur less readily and less extensively in the particles isolated from shocked dogs (Fig. 3). *Enzymatic data.* Fig. 4 shows that the oxidation of succinate, malate and α-ketoglutarate was essentially unimpaired even after 5 hours of shock; in many experiments the 2 curves were superimposable. Experiments with acetate and butyrate were also negative. No requirements for coenzyme I, guanosine diphosphate¶ or cytochrome c could be demonstrated; in fact, added coenzymes often were found to be inhibitory. On the other hand, Table I indicates that in the presence of the hexokinase system oxygen uptake and phosphate esterification were often decreased: thus in seven experiments with α-ketoglutarate the normal consumed an average of 11 microatoms of oxygen per mg nitrogen and 22 micromoles of phosphate resulting in a P/O of 2.0; this was reduced in shock to 7.5 microatoms oxygen and 12 micromoles phosphate

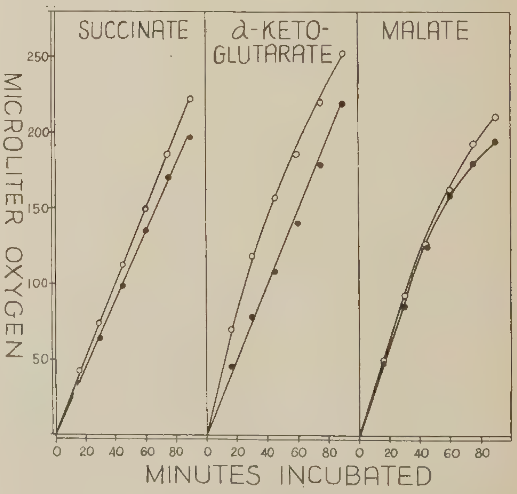

FIG. 4. Oxygen uptakes of heart mitochondria from normal and shocked dogs. Main compartment: 20 μM $MgCl_2$, 3 μM ATP, 50 μM phosphate buffer, pH 7.5, 40 μM substrate, mitochondria (app. 1 mg N) and 0.5 M sucrose to 2.0 ml; center well: 0.2 ml 2 N NaOH; temperature: 30°C; gas phase: oxygen. ○ = normal, ● = 5 hr shock.

¶ Generously presented to us by Dr. D. M. Gibson of the Enzyme Institute.

giving an average P/O of 1.6.

Discussion. It may be concluded, therefore, that the mitochondria are structurally altered in hemorrhagic shock and that these *in vivo* changes differ from the *in vitro* degenerations described by Harman, for in the formalin-fixed preparations the basic rod shape is maintained despite the swelling and distortion.

Upon isolation in sucrose solution, however, the serial transformations observed by Harman are found to take place, *i.e.* the rods change into spheres which increase in size and ultimately form "crescents." Under these conditions the spheres derived from the abnormal mitochondria are smaller and exhibit less of a tendency to swell and to turn into "crescents." This phenomenon suggests that shock alters the particles in a way which renders them less responsive to environmental conditions. It will be of interest to study their submicroscopic internal structure.

The observation that the *in vitro* oxidative activities of the abnormal mitochondria are affected by the presence of the hexokinase system has raised an interesting point. It is possible that the classical Warburg methods, which supply an optimal environment as far as cofactors and substrate requirements are concerned, obscure or reverse early enzymatic defects. The negative results of Fig. 4 could thus be explained. In the living organism, on the other hand, the mitochondria are called upon to furnish an undiminishing supply of energy and building materials for use by other cell constituents. It may well be that the hexokinase trap which sets up a constant extramitochondrial demand for ATP mimics to some extent such *in vivo* situations. The results of the enzymatic studies reported here would then indicate that the heart mitochondria of shocked dogs are capable of generating enough energy to maintain themselves in a functional state but begin to fail when exposed to stress conditions which force them to produce ATP at rates beyond those which will suffice for their maintenance. Future work will have to elucidate the correctness of this hypothesis.

Summary. Dogs were subjected to irreversible hemorrhagic shock. Their hearts, livers, and kidneys contained mitochondria which differed in size and shape from those of normal dogs. Isolated by differential centrifugation the mitochondria from shocked animals were found to retain the normal oxidative capacities for Krebs cycle substrates but tended to have impaired activities in the presence of the hexokinase system.

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Some Factors Influencing Potentiation of Cardiac Contractility Following an Extrasystolic Beat.* (22360)

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The increase in contractility of cardiac muscle following an extrasystole was first described by McWilliams(1). Cattell and Gold (2,3) have confirmed and extended this finding and Garb and Penna(4) have determined the effects of the interval between the basal and extrasystolic beats on the potentiated contraction in cat papillary muscle. The present report deals with some of the factors influencing post-extrasystolic potentiation (PEP) of cardiac contractility as determined in the isolated rabbit auricle.

Methods. The isolated quiescent rabbit auricle suspended in Tyrode solution was used. Details of this preparation and the Tyrode solution used are found in a publication by Rosin and Farah(5). The bath temperature was controlled at 37.0-37.5°C. Electrical stimulation of the auricle was accomplished by means of 2 coupled Grass stimulators (Model S4C). The first stimulator was used for driving at basal rates and served to trigger the second stimulator which provided the extrasystolic beats. The interval between basal and extrasystolic beat as well as voltage and pulse duration could be varied at will. Extrasystolic stimuli were interjected through the same electrodes which delivered the basal stimulus or at a point 2 to 4 mm from the latter electrodes. Results in both instances were essentially the same. Contractility was recorded by means of a Grass force transducer and a Grass inkwriting electroencephalograph. Quinidine sulfate, when used, was added to the bath in a dosage of 0.25-1.0 mg% and readings were made 30 minutes after addition of the drug.

Results. Post-extrasystolic potentiation of cardiac contractility could be demonstrated both in the isolated auricle and isolated ventricular strips of the rabbit. The quantitative data to be reported have been obtained on the quiescent isolated rabbit auricle stimulated at basal rates of 0.2 to 5 beats per second.

Fig. 1A illustrates a typical result. It can be seen that a single extrasystolic beat produced a potentiation of contractility which was increased by interjecting a second and a third extrasystolic beat. This cumulative effect of extrasystolic beats has been regularly observed and usually reached a maximum following 5 to 8 such stimulations. Maximum potentiation of cardiac contractility thus attained exceeded 100 and sometimes attained 300 to 400%. The contraction velocity of the post-extrasystolic beat is accelerated as the peak of the contraction occurs only slightly later than that of the pre-extrasystolic beat (Fig. 1B).

It is difficult to state whether the electrical extrasystole which produced potentiation also produced a mechanical extrasystole. In many of our experiments (see Fig. 1A) there were no readily visible mechanical extrasystoles. However, close inspection of some of the tension curves frequently showed a slight irregularity in the descending part of this curve which may indicate a mechanical change. With the recording methods used it is impossible to decide whether an electrical or both electrical and mechanical extrasystole were essential for the production of potentiation. It is hoped to resolve this problem and such studies are now being initiated.

The effect of resting tension on the production of PEP was studied. In general PEP was observed with low or high initial tensions. Usually an increase of the initial tension by 1 to 2 g increased the height of the basal beat as well as PEP. On occasion an excessive increase in tension would reduce both the basal beat and the PEP. This is in conformity with

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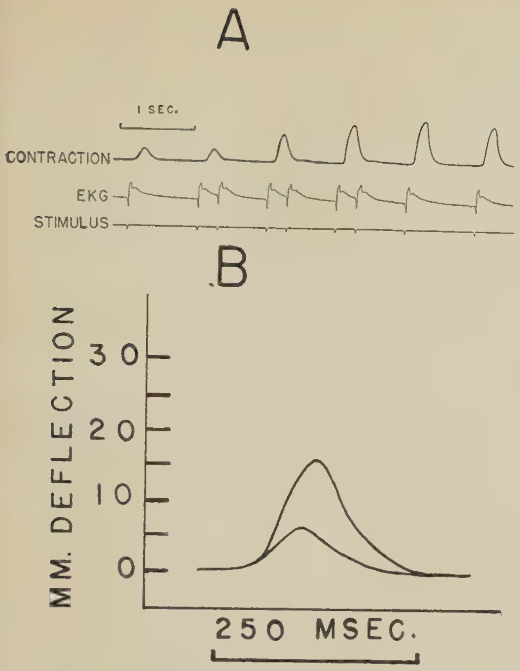


FIG. 1A. Effect of 3 extrasystoles on contractions of isolated left auricle of rabbit. Temp. 37.5°C. Tyrode solution. Note increase in contractile tension following interjection of the extrasystoles.

FIG. 1B. Superimposed records of contractions obtained from isolated left auricular preparation. Lower trace: basal contraction; upper trace: contraction following a single extrasystolic stimulus delivered 80 milliseconds after basal stimulus. Basal rate 2 per sec. Temp. 37°C.

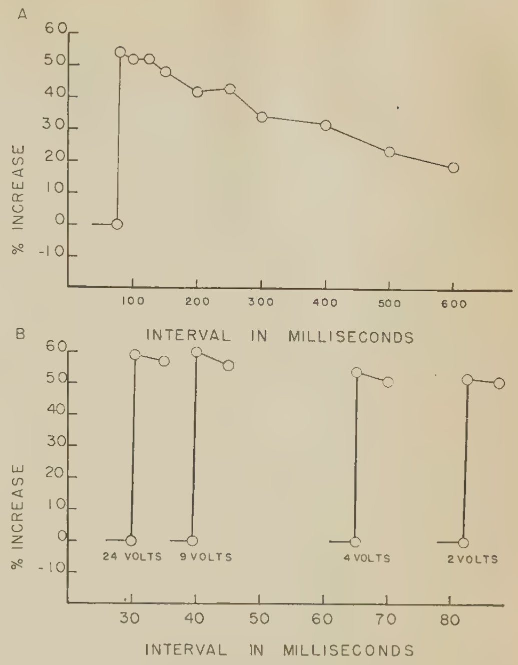


FIG. 2A. Effect of interval between basal and extrasystolic beat on post-extrasystolic potentiation in rabbit auricle. Basal rate = 1 per sec. Temp. 37°C. Height of first post-extrasystolic beat expressed as % of basal beat. Stimulus 15 volts.

FIG. 2B. Effect of stimulus intensity on PEP in rabbit auricle. Conditions as in Fig. 2A except for stimulus intensity.

Starling's law of the heart. The PEP was not accompanied by a measurably different action potential from that of the basal beat (Fig. 1A). In general, the first post-extrasystolic beat showed maximal potentiation, however, the second, third, and even fourth beats following a single extrasystolic beat would show some potentiation. The decay of potentiation followed an exponential curve and in this respect our results confirm those of Cattell and Gold(3), Garb and Penna(4), and Rosin and Farah(5).

The interval between the extrasystolic beat and the post-extrasystolic beat was varied between 1 and 120 seconds. It could be shown that PEP was still apparent after a rest period of 120 seconds. In this respect the results are qualitatively similar to those of Garb and Penna(4) and Rosin and Farah(5) who determined the effect of rest period on post-extrasystolic potentiation, and post-stimulation potentiation of cardiac muscle respectively.

The effects of the interval between basal and extrasystolic beats on PEP were studied in 25 auricles. In general the shorter the interval the greater the PEP until an interval was reached when the auricle would not respond (Fig. 2A). If a threshold stimulus was used the interval necessary to produce maximal potentiation was longer than when supramaximal stimuli were used (Fig. 2B). The shortest possible interval could be obtained by using stimuli equal to 6 to 9 times threshold and this minimal interval corresponded to the effective refractory period, as determined from a strength-interval curve. In most experiments maximal potentiation was seen when the interval between basal and extrasystolic beat was minimal. However, in some experiments maximal potentiation did not

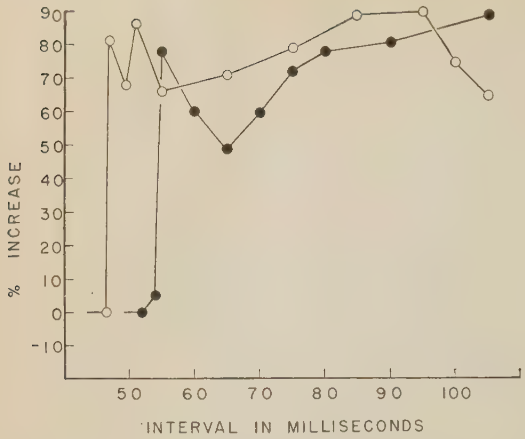


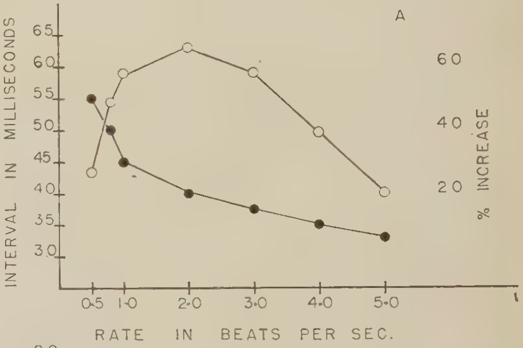
FIG. 3. Effect of interval between basal beat and extrasystole on 1st post-extrasystolic beat showing atypical response curve. Rabbit auricle. Temp. 37°C.

correspond to the effective refractory period but occurred at longer intervals (Fig. 3). We do not have any explanation for this variation, and it was observed only in a few preparations; however, these changes may correspond to the dips in the excitability curve described by Brooks, *et al.*(6).

Since rate of stimulation influences the refractory period of cardiac muscle, we have determined the effects of rate on the PEP in the isolated rabbit auricle. It could be shown that an increase in rate produced a shorter effective refractory period as determined by strength-interval curves and decreased the time interval between the basal and extrasystolic beat necessary to produce a maximal potentiation (Fig. 4A). The observation was made that an increase in rate of stimulation not only decreased the time interval for PEP but also increased the extent of potentiation (Fig. 4B) up to rates of about 3 per second; at higher rates both basal and potentiated beats were reduced. This finding is different from that of Garb and Penna(4) who did not find this relation of rate of stimulation of cat papillary muscle to the magnitude of the PEP.

At high rates of stimulation (4.0-5.0/sec.) the potentiation would occasionally be delayed, *i.e.*, no potentiation of the first post-extrasystolic beat but marked potentiation of the second would be observed. Garb and Penna(4) and Rosin and Farah(5) have also observed this delayed potentiation which occurs only at high rates of stimulation.

Quinidine is known to prolong the effective refractory period of cardiac muscle. We have thus determined the effects of quinidine on the interval necessary for the production of PEP as well as the extent of the potentiation produced. Following control readings quinidine was added in increasing cumulative concentrations to the bath and both strength-interval curves and PEP were determined. The data are given in Fig. 5 and it can be seen that quinidine in concentrations of 0.25 to 1.0 mg % prolonged the interval necessary to



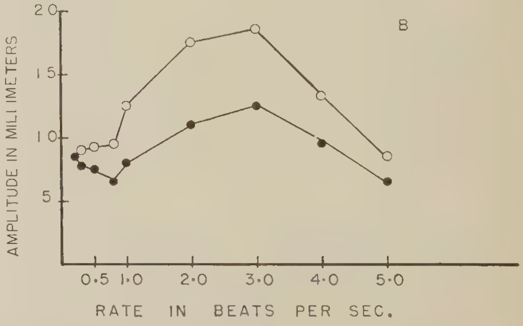


FIG. 4A. Effect of rate of basal stimulation on minimal interval between basal beat and extrasystolic which produced post-extrasystolic potentiation in rabbit auricle. Temp. 37°C. ●—● Minimal interval between basal beat and extrasystole producing potentiation of the first PEP. ○—○ % change in tension of PEP as related to basal beats at each rate.

FIG. 4B. Effect of rate of basal stimulation on post-extrasystolic potentiation. Avg of 4 auricular preparations. Temp. 37°C. Post-extrasystolic potentiation measured at minimal interval between basal and extrasystolic beat producing potentiation. Abscissa: Basal rate. Ordinate: Tension of isometric twitch expressed in millimeters of pen deflection. ●—● Basal beat. ○—○ Potentiated beat.

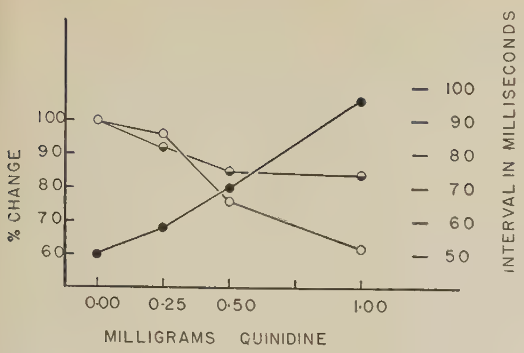


FIG. 5. Effect of quinidine on interval between basal beat and the extrasystole which produced maximal PEP in isolated rabbit auricle. Temp. 37°C. ●—● Minimal interval in milliseconds between basal beat and extrasystole which produces maximal PEP. *Half-solid circles*—Contractility at basal rate (1/sec.) expressed as % of control. ○—○ Change in PEP expressed as % of control.

produce PEP. Quinidine decreased the extent of the potentiation but also decreased the height of the basal contraction. However, the per cent decrease in the potentiated beat exceeded the changes in the basal beat (Fig. 5). It is thus likely that quinidine in some way interfered with the process or processes responsible for the initiation of post-extrasystolic potentiation.

Discussion. The presence of post-extrasystolic potentiation (PEP) of cardiac beats was demonstrated in both isolated auricular and ventricular muscle strips of the rabbit. This phenomenon of PEP has been observed by others in isolated cardiac muscle preparations (1-4). In our own experience PEP occurs in the isolated heart of dogs, rabbits and cats perfused by the Langendorff technic. Furthermore, PEP has been observed in occasional auricular and ventricular strips of the rabbit when spontaneous extrasystoles occurred. In the dog heart-lung preparation we have observed that the appearance of one or more extrasystoles would significantly improve the cardiac function especially if the heart showed signs of decompensation. The effects of such extrasystoles became apparent by a temporary decrease in the right auricular pressure and cardiac size, a temporary increase in cardiac output and stroke volume. All these changes have been frequently observed but have been explained on the basis of changes in cardiac filling and "Starling's law of the heart." The observations in the heart-lung preparation are difficult to analyze since changes in fiber length occur and thus the relation of PEP to changes in fiber length cannot be clearly formulated. Recently Siebens, *et al.*(7) studied this phenomenon in the dog heart and changes in fiber length of the ventricle could be excluded as a cause of post-extrasystolic potentiation. It is thus likely that the PEP studied in the isolated auricular and ventricular strip is not characteristic of the isolated preparation alone but also occurs in blood-perfused, intact hearts.

The relation of post-extrasystolic potentiation to post-stimulation potentiation and to the staircase (Treppe) phenomenon should be considered. In our experience the behavior of these 3 phenomena responds to physical as well as pharmacological changes in the same directions and it is likely that the underlying fundamental mechanisms for all 3 types of potentiation are the same. As regards the mechanism of post-extrasystolic potentiation little can be said at this time. Ellis(8) has suggested that glucose-phosphate is related to the staircase phenomenon. Recently Ritchie and Wilkie(9) have concluded that the staircase phenomenon and post-tetanic potentiation in frog striated muscle were caused by the delay in the fall of the active state of muscle postulated by Hill(10). It is possible that the active state of muscle is related to post-extrasystolic and post-stimulation potentiation. The active state persists for milliseconds while the potentiation of contractility persists for several beats and can be elicited minutes after the extra-systolic beat. These properties of PEP and post-stimulation potentiation make it difficult to explain these phenomena solely on the basis of a prolongation of the active state. A process which is only slowly reversible and which is dissipated by the following contractions may precede the active state and may determine the length of the active state and velocity of contraction.

The PEP of auricular muscle is related to the refractory period since an increase or decrease in the refractory period also produced parallel changes in the PEP.

Summary. Post-extrasystolic potentiation (PEP) of cardiac contractility was studied in rabbit auricular muscle. It is shown that potentiation is related to the interval between basal beat and extrasystolic beat. An increase in driving rate up to about 3 per second increased the extent of potentiation and decreased the interval between basal beat and the extrasystole necessary for the production of maximal potentiation. Quinidine increased the effective refractory period and the interval for producing PEP and decreased the degree of potentiation produced by the extrasystole.

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Metabolic Actions of Acetic Acid Analogs of Thyroxine and Triiodothyronine.[*] (22361)

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In the field of thyroxine analogs and possible derivatives which may have special metabolic characteristics, the most striking recent observations were those of Gross and Pitt-Rivers(1) and of Roche, Lissitzky and Michel(2) that 3,5,3′-triiodothyronine (Trit) was several times more active than thyroxine. Many workers have since confirmed this on a variety of test animals(3). The time of delay before the metabolic effects of Trit are seen is now recognized as shorter than with thyroxine(4-7), but still present. The duration of Trit action is also more brief, effects which are more striking in the myxedematous human than in experimental animals(8,9).

Thibault and Pitt-Rivers(10,11) reported that 3,5,3′,5′-tetraiodothyroacetic acid (Tetrac) and 3,5,3′-triiodothyroacetic acid (Triac) produced an immediate *in vitro* stimulation of kidney and liver slices appearing at concentrations about 0.01 µg Triac per ml or 0.05 µg Tetrac per ml and reaching a maximum at respectively, 1.0 and 5.0 µg per ml. In addition, Tetrac was stated to cause a considerable rise in metabolic rate of thyroidectomized rats in 2 hours followed by a return to the starting level after 4 hours. Pitt-Rivers had previously reported on the activity of Tetrac and Triac(12), noting no unusual results except for unexpectedly high toxicities. The present report concerns possible qualitatively distinct properties of Tetrac and Triac.[†]

Methods. All rats used were from the Sprague-Dawley strain, thyroidectomized at least 6 weeks previously. Most tissues were prepared as previously discussed(13). Ehrlich mouse ascites tumor cells, cultured *in vivo* in Carworth white mice,[‡] were washed sev-

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[*] This work was supported by grants from the Council on Pharmacy and Chemistry of American Medical Assn., the Smith, Kline, and French Foundation, and Committee on Growth of American Cancer Society.

[†] Samples of Tetrac and Triac were obtained through the generosity of Dr. Pitt-Rivers and of Dr. John A. King, Warner-Chilcott Laboratories.

[‡] These cells from a Southern Research Institute culture were obtained through the courtesy of Dr. Warner W. Carlson and Mr. J. T. Huggins.

TABLE I. Effect of Triac or Tetrac Added *In Vitro* to Tissue Slices Prepared from Thyroidectomized Rats.

Compound added, µg/ml	No. of animals	% of control Q_{O_2}					
		Kidney		Liver		Heart	
		0–15	0–60	0–15	0–60	0–15	0–60
Triac: .01	8	99.3	100.0	102.0	103.5	115.0	111.4
.10	8	97.2	100.0	101.7	102.3	107.5	111.1
1.0	8	94.1	97.3	97.1	93.7	104.9	110.4
5.0	2	97.9	99.0				
10.0	8	95.2	96.1	93.7	89.4	85.7	84.7
Tetrac: .05	8	87.8	92.7	93.8	88.3		
.50	8	96.9	97.1	101.4	98.7	96.4	100.8
1.0	2	103.6	100.9				
5.0	8	95.7	97.8	105.5	102.7	86.7	98.3
10.0	2	98.2	95.3			77.4	91.7
50.0	8	73.4	65.1	101.0	101.8	52.7	55.7

eral times by suspension in Krebs' Ringer solution followed by mild centrifugation. A 10% v/v suspension was made from the final packed tumor cells using a solution appropriate to the determination of oxygen consumption (Krebs' Ringer-phosphate) or glycolysis (Krebs' Ringer-bicarbonate). Any thyroxine analog being studied was present in 0.005 N NaOH final concentration. Control vessels always contained the same amount of alkali and 0.9% NaCl.

Results. Findings are divided into 3 groups: (1) *In vitro* effects on rat tissues, (2) *In vivo* injection into thyroidectomized rats, (3) *In vitro* effects on tumor cells.

1. *In vitro Triac and Tetrac effects on tissue metabolism.* Table I shows oxygen consumption values calculated as percentages of control values for 0-15 and 0-60 minutes of observation. Liver, kidney and heart were not stimulated significantly over these time intervals. Kidney was clearly depressed by 50 µg Tetrac/ml solution and heart by both 10 µg Triac/ml and 50 µg Tetrac/ml. Heart also appeared to have been inhibited the first 15 minutes by 5 and 10 µg Tetrac/ml, but escaped by 1 hour. Liver metabolism was not altered markedly by either material, although it was slightly depressed by the highest level of Triac. Readings actually were continued for a second 60 minutes, with no significant changes beyond those already discussed. Rates of shaking of the apparatus were also varied over the range of 80 to 120 per minute. The data published by Thibault and Pitt-Rivers(10) for kidney yield the following averages: with 5 µg Tetrac/ml solution, 73% increase for 15 minutes and 30% for 60 minutes; with 1 µg Triac/ml, 106% and 30% for 15 and 60 minutes, respectively. In no instance did the results obtained in this laboratory approach these increases. The later report by these workers(11) indicated a more evanescent effect of Triac on liver slices. Concentrations from 0.01 to 1.0 µg/ml all gave increases averaging 28% for 15 minutes and 7% over 60 minutes.

2. *In vivo effects of injected Triac and Tetrac.* The reported immediate increase of BMR in thyroidectomized animals produced by Tetrac injection(10) might be due to undesirable non-specific effects. We have studied the metabolism of tissues removed from thyroidectomized rats 1.5 hours after injection of 2 mg Tetrac/kg or 1 mg Triac/kg. The tissues were immediately chilled to 5°C and kept at this temperature throughout the period of preparation, about 0.5 hour. The effective time after injection is considered to include only the 15 minute equilibration time at 37° in addition to the 1.5 hours after injection.

The results in Table II fail to show any rapid response following injection of the acetic acid analogs into thyroidectomized animals. Heart was included because it has been found to respond with such high sensitivity to injected thyroxine and skeletal muscle because it makes up so much of the metabolizing body mass. Preliminary experiments with liver and kidney have shown the same absence of immediate energy metabol-

TABLE II. Oxygen Consumption of Tissues Removed from Thyroidectomized Rats Injected with Tetrac (2 mg/kg) or Triac (1 mg/kg).

	% of control Q_{O_2}					
	Tetrac-injected			Triac-injected		
Hr after inj.*	Heart	Diaphr.	Skel. M.	Heart	Diaphr.	Skel. M.
1¾–2	94.9	94.4	109.2	102.8	97.8	94.7
2 –2¼	106.9	96.1	105.3	97.9	93.4	93.4
2¼–2½	96.3	90.0	104.6	98.1	102.9	90.8
2½–2¾	92.3	91.9	113.6	100.3	97.3	95.5
2¾–3¼	111.1	94.9	105.0	92.2	96.6	88.3
3¼–3¾	88.0	92.5	100.0	89.5	98.1	83.6

* Not including approximately ½ hr required for preparation of tissues, during which time tissues were kept at 0°C. Four animals used in each category.

TABLE III. Metabolism of Tissues Excised from Thyroidectomized Rats Injected with Thyroxine, Tetrac or Triac.

		% increase above control Q_{O_2}		
Tissue	Thyroid-ectomized Q_{O_2}	L-thyroxine*	Tetrac*	Triac*
Liver	.98	59.2	53.1	52.0
Diaphragm	.72	34.7	23.6	33.3
Heart	.44	170.6	147.7	277.3
Skel. M.	.68	27.9	17.6	23.5
Pancreas	.83	39.8	20.5	25.3
Saliv. gl.	1.86	31.7	22.0	20.4
Kidney	3.27	35.5	21.7	45.9

* Compound specified in each case was inj. at a dose of 2 mg/kg body wt/day for 4 days and animal sacrificed on fifth day. Three animals in each group.

ism change.

Thyroidectomized rats were also injected with Triac and Tetrac at several dose levels once per day for 4 days, and sacrificed on 5th day. The most consistent responses were obtained at a level equimolar to 2 mg thyroxine/kg/day. These are shown in Table III, together with responses to L-thyroxine.§ The cumulative effect from these materials, as with thyroxine, suggests that metabolism is stimulated for longer than 2 to 4 hours. It may be estimated that Tetrac is about three-quarters as active as L-thyroxine. Since Triac stimulated tissues to approximately the same extent as thyroxine, it is about one-fourth as active as L-triiodothyronine. The apparently greater effect of Triac on the heart requires further study.

3. *In vitro responses of Ehrlich's mouse ascites tumor cells.* This tumor cell appears to be a useful test object with Triac and Tetrac(14). Table IV gives the Q_{O_2}, $Q_{CO_2}^{O_2}$ and $Q_{CO_2}^{N_2}$ values obtained in terms of the wet weight of tumor tissue. Since the dry weight averaged 11.0% of the wet weight, multiplication by a factor of 9.1 yields dry weight Q's. The endogenous metabolic rate was the same as that with pyruvate, but glucose caused a 40% decrease. The nature of the foodstuff used in the absence of added substrate is not known, but the repeated washings with saline should have removed such substances as pyruvate or lactate. As is typical of tumor cells, addition of glucose caused a tremendous increase in glycolysis over the non-nutrient, about 12-fold for aerobic and 32-fold for anaerobic.

The tumor cell Q_{O_2} was not affected by Triac from 0.01 to 10 μg/ml, or Tetrac 0.05 to 5, except for 8-10% increases at levels of 0.5 to 1.0 μg/ml. Triac at 50 and 100 μg/ml and Tetrac at 50 showed increasing inhibition. In contrast, as shown in Fig. 1 and 2, both aerobic and anaerobic glycolyses were enhanced by 50 μg Triac/ml, the former by 52% and the latter 22%. Somewhat lesser effects were shown by 60 μg Tetrac/ml. However, the thyroxine isomer, B-5522, N-(3,5-diiodo-4-hydroxybenzoyl) -3,5-diiodotyrosine, devoid of thyroxine-like, metabolism-stimulating properties when injected into thyroidectomized animals, also gave increases in both aerobic and anaerobic glycolyses. In addition, thyroxine itself accelerated aerobic glycolysis and Trit anaerobic.

In general, these results confirm the observations of Heimberg *et al.*(14) on the *in*

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§ Sodium L-thyroxine was kindly provided by Dr. H. L. Fevold, of the Travenol division of Baxter Laboratories.

TABLE IV. Oxygen Consumption, Aerobic Glycolysis and Anaerobic Glycolysis of Ehrlich Mouse Ascites Tumor Cells.

			Triac, μg/ml (as % of control)						Tetrac, μg/ml (% of control)			
Determ.	Substrate	Control	.01	.10	1.0	10	50	100	.05	.5	5.0	50
Q_{O_2}	None	.90				90.0	33.3	10.0				
	Glucose	.56	97.0	98.0	110.1	98.2	41.1	16.1	102.0	108.1	99.0	45.5
	Pyruvate	.98				93.9	55.1	22.4				
$Q_{CO_2}^{O_2}$	None	.14										
	Glucose	1.75				104.6	152.0	87.4				
$Q_{O_2}^{N_2}$	None	.08										
	Glucose	2.59	102.5	99.5	95.8	113.5	121.6	70.2	100.0	99.3	101.5	

vitro stimulation of glycolytic activity by Tetrac and Triac. However, our findings that thyroxine and Trit have similar effects throws considerable doubt on interpreting these changes as being specifically related to an action of the iodothyroacetate derivatives. Furthermore, the comparable activity exhibited by a physiologically inactive thyroxine isomer, B-5522, suggests that the entire phenomenon is related more generally to some structural configuration of these compounds (such as the 3,5-diiodo-4-hydroxyphenyl group) than is the complete oxidative pathway. It would be quite premature to insist that one function or the other was the more "genuine" action of the thyroid hormone.

The present studies seem to put the acetic acid analogs of thyroxine and Trit into a rather less dramatic relationship to the "physiologically active" thyroid hormone than was indicated by Thibault and Pitt-Rivers. Both Triac and Tetrac are thyroactive compounds, but show no clearly qualitative differences from thyroxine or, more appropriately, Trit. Other studies being carried out at present indicate that Triac, Tetrac and Trit may all produce a more rapid response on the sensitive heart than thyroxine when injected into thyroidectomized animals. Whether the acetic acid analogs have, also like Trit, a more rapid turnover, remains to be determined.

If Trit is, as has been suggested, the "active" form of the thyroid hormone produced from thyroxine, it would seem unlikely that it is transformed into Triac prior to exerting its final actions. An alternate interpretation of these interconversions might be that any compound with the proper configuration is potentially active in its own right and that metabolism of thyroxine may yield a series of compounds, including Trit, Tetrac, Triac, and other derivatives.

Summary. Tetraiodothyroacetic acid (Tetrac) and triiodothyroacetic acid (Triac), in-

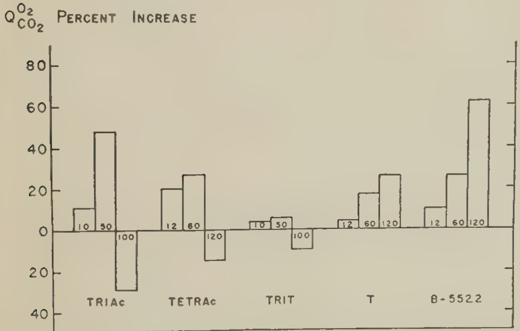

FIG. 1. Aerobic glycolysis of Ehrlich mouse ascites tumor cells as affected by thyroxine, Trit and related materials at various concentrations, shown as μg/ml.

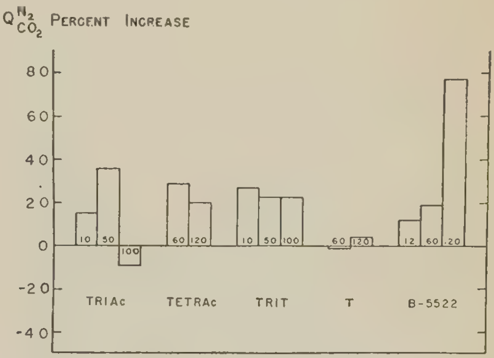

FIG. 2. Anaerobic glycolysis of Ehrlich mouse ascites tumor cells as affected by thyroxine, Trit and related materials at various concentrations, shown as μg/ml.

jected into thyroidectomized rats over a period of several days, have the same order of activity as an equimolar dose of L-thyroxine, as judged from the oxidative metabolism of excised tissues. Triac and Tetrac do not appear to produce an immediate metabolism-stimulating effect, either injected into animals or incubated *in vitro* with tissue slices. At levels which depress oxygen consumption of Ehrlich mouse ascites tumor cells, Triac and Tetrac enhance aerobic and anaerobic glycolyses. However, this effect is not considered specific to the acetate analogs, since thyroxine, triiodothyronine, and a physiologically inactive thyroxine isomer all are capable of producing similar changes.

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Radiation-Induced Alteration of Fibrinogen Clotting Rate and Clot Lability.* (22362)

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Studies of the effect of radiation on the biological activity of proteins have been confined almost exclusively to enzymes. In the case of the latter, an important body of information leading to a better understanding of radiation effects has been obtained(1). Investigations of proteins exposed to ionizing radiation have yielded a wealth of physicochemical data which must, however, be supplemented by examination of the radiation-induced modifications of their biological functions. In the light of the extensive work that has already been done on purified fibrinogen, it was decided to investigate the effects of x-radiation of fibrinogen on its subsequent clotting by thrombin. Fibrinogen has already been studied physicochemically after irradiation in the dry state(2) and in concentrated solution(3). In these studies, the doses of radiation employed were of such high magnitude as to have little practical significance from the point of view of mammalian radiation damage. The present study is concerned with the influence of low doses of radiation.

Materials and methods. Purified bovine fibrinogen (lot No. 55199) was generously supplied by Dr. E. C. Loomis of Parke, Davis and Co., and was shown to contain 90-93% clottable protein. Solutions of this fibrinogen were prepared in 0.15 M NaCl buffered with

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* This work was performed under U. S. Atomic Energy Commission contract AT(30-1) 1554, administered by L. V. Heilbrunn.

imidazole at pH 7.4 (final buffer concentration 0.015 M). Unless otherwise stated, the fibrinogen concentration in all experiments was 3 mg/ml. Single x-ray doses were given to freshly prepared fibrinogen solutions in Lusteroid tubes. The author wishes to express his gratitude to Messrs. J. L. Weatherwax and L. Stanton of the American Oncologic Hospital for kindly making available to him their x-ray facilities. The conditions of irradiation were 140 kilovolts, 5 milliamperes, 3 mm half-value layer filtration, and 20 cm target distance. The output of the machine was 150 r per minute. The clotting time, *i.e.*, the time required to attain firm-gel endpoints, of irradiated and control solutions of fibrinogen, was determined in triplicate upon the addition of 0.05 ml of saline solution containing, unless otherwise stated, 0.063 unit of bovine thrombin (Topical, Parke, Davis). The clotting reaction took place in 12 x 75 mm Pyrex tubes contained in a water bath at 37.5°C. After allowing the fibrin clots formed in the above manner to remain in the water bath for at least one hour, an equal volume of 10 M urea was added. Each tube was shaken 3 times in order to free the clot from the bottom of the tube. The time for complete dissolution of the clot was then determined. In the experiments with sulfhydryl reagents the latter were added to the fibrinogen solutions prior to irradiation.

Results. Roentgen irradiation increased the clotting time of fibrinogen. The data are given in Fig. 1 and 2 where the logarithm of clotting time is plotted against the radiation dose. In Fig. 1 the dose range is from 0 to 500 r in steps of 100 r; each line represents a separate experiment. In Fig. 2 the dose range is 0 to 100 r in steps of 25 r, and here the 3 lines represent 3 different amounts of thrombin added. In both figures a linear relationship was obtained between the logarithm of the clotting time and the x-ray dosage. Inspection shows that the linear relationship between 0 and 100 r in Fig. 1 is verified by the data in Fig. 2. Fig. 3 indicates that the difference in clotting time between unirradiated and irradiated fibrinogen (500 r) varies inversely with the thrombin concentration. When 0.5 unit of thrombin are added, the difference in clotting time between the unirradiated and the irradiated solution is negligible. Thus, the radiation-induced clotting delay can be prevented by adding an excess of thrombin. The results of irradiation with 500 r on the clotting time of various concentrations of fibrinogen (1 mg/ml to 5 mg/ml) are plotted in Fig. 4. The clotting delay due to radiation was found to be independent of the fibrinogen concentration.

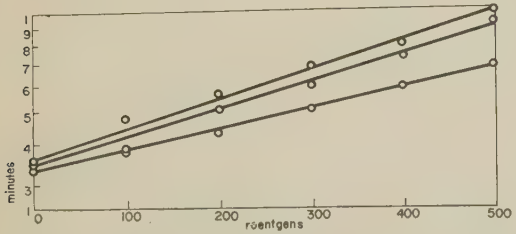

FIG. 1. Clotting time of fibrinogen as a function of radiation dose. Each curve represents a separate experiment.

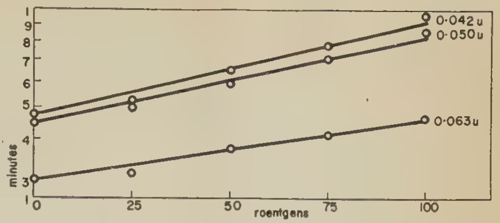

FIG. 2. Clotting time of fibrinogen as a function of radiation dose, using 0.063, 0.050, and 0.042 unit of thrombin, respectively.

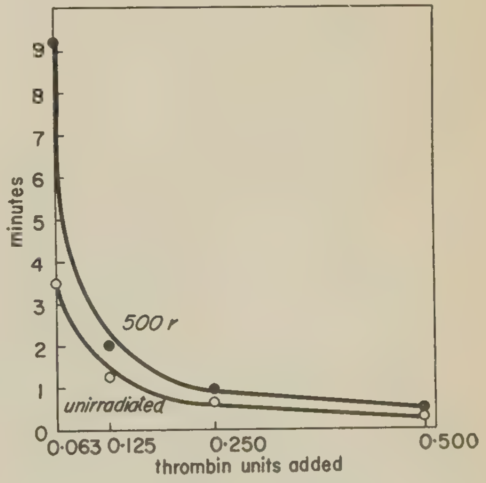

FIG. 3. Clotting time of fibrinogen as a function of thrombin concentration.

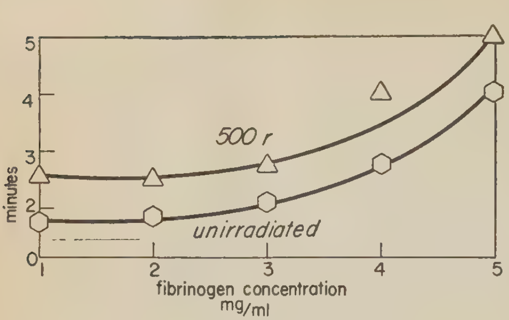

FIG. 4. Clotting time of fibrinogen as a function of fibrinogen concentration.

The extent to which the time of solution in 5 M urea of fibrin clots obtained from irradiated fibrinogen is shortened is indicated by Fig. 5. The plots of time of solution against x-ray dose, at 3 different concentrations of thrombin, reveal that when 0.25 unit of thrombin are added, there is no significant change. When the amount of added thrombin is decreased to 0.125 unit or to 0.063 unit, the time of solution decreases markedly as the x-ray dose increases. Thus, at thrombin concentrations where x-rays prolong the clotting time (Fig. 3), the time of solution of fibrin clots in urea is also decreased.

Addition to fibrinogen, prior to irradiation, of sulfhydryl compounds yielded the results summarized in Table I. 10^{-3} M cysteine or glutathione failed to abolish the radiation-induced clotting delay; they prolonged the clotting time in both the irradiated and unirradiated solutions. Thioglycollic acid (10^{-3} M), as well as higher concentrations of cysteine (10^{-2} M) completely inhibited the clotting process in both the irradiated and control solutions.

Discussion. The radiation-induced clotting delay in fibrinogen solutions may be attributed to a change in the fibrinogen molecule itself. That it is not due to a destruction of the latter will be reported in a subsequent communication, for irradiation failed to alter the amount of clottable protein. Indeed, even with the exposure of fibrinogen solutions to doses up to 130 kiloroentgens, Scherage and Nims (*op. cit.*) found practically no decrease in fibrin yield upon the addition of thrombin. They found that high doses of x-rays altered the fibrinogen to produce a polydisperse material with a higher sedimentation constant and increased viscosity. With the low doses of x-rays employed in the present study no change in viscosity was noted. Consequently, the radiation-induced alterations in the fibrinogen molecule exposed to low doses are of a much smaller magnitude than the effects observed by Scherage and Nims. Nevertheless, the effect of irradiation with low doses sufficed not only to delay the clotting time of fibrinogen, but also to produce structural alterations of the fibrin molecule as revealed by the decrease in time to dissolve fibrin clots in concentrated urea solution.

TABLE I. Effect of Sulfhydryl Reagents on the Clotting Time of Unirradiated and Irradiated (500 r) Fibrinogen.

Additions	Conc. (M)	Control	Irrad.
None		4′ 30″	7′ 40″
Cysteine	8×10^{-3}	11′ 00″	18′ 10″
	4×10^{-2}	$\propto$	$\propto$
Glutathione (SH)	3×10^{-3}	8′ 15″	12′ 45″
Thioglycollic acid	2×10^{-3}	$\propto$	$\propto$

The progressive increase in clotting time due to x-radiation, leading finally to the establishment of an altered fibrin gel, could mean either that there has been a change in the initial polymerization of the activated fibrinogen, or that a modification, present in the fibrin strands, so changes the further polymerization as to give rise to an altered structural framework of the final clot. Both alternatives are probably at work to produce

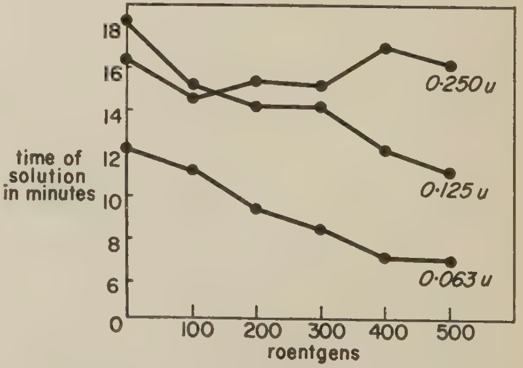

FIG. 5. Time of solution in 5 M urea of fibrin clots obtained from irradiated fibrinogen (500 r) clotted with 0.250, 0.125, and 0.063 unit of thrombin, respectively.

the results obtained. That at least the first possibility is involved can be inferred from the fact that, at high thrombin concentrations, the clotting delay due to radiation is no longer evident. At high thrombin concentrations there is an increase in the rate at which activated fibrinogen appears in solution(4). Apparently, at a high concentration of thrombin, the elaboration of normally activated fibrinogen is sufficiently rapid to overcome the radiation-induced reduction of polymerization. The increase in the difference in clotting time between the irradiated and the control solutions as the thrombin concentration is decreased is then a reflection of the decreased amounts of normally activated fibrinogen formed, the latter being replaced by increasing amounts of radiation-altered material. This modification of the fibrin strands produces an altered clot structure, since these strands comprise the network of a clot which is more highly accessible to the action of urea. At a relatively high thrombin concentration, this effect is also abolished, and this favors the interpretation that the primary modifications produced by radiation become manifest in the early stages of the clotting process.

The effect of low doses of radiation on fibrinogen is apparently not due to an oxidation of sulfhydryl groups, for as pointed out previously, sulfhydryl reagents failed to abolish the radiation-induced clotting delay. Nevertheless, these findings do not exclude the possibility that the polymerization phases of the clotting process are sulfhydryl dependent, since cysteine, glutathione, and thiourea protected radiation-sensitive sites in the fibrinogen molecule at doses ranging from 100 to 600 kiloroentgens(3). The persistence of clotting delay in the presence of similar concentrations of some of the same reagents indicates, however, that sulfhydryl reagents and x-rays act on separate sites in the fibrinogen molecule; both of these sites are essential for clotting to take place.

Summary. Purified bovine fibrinogen was exposed to single x-ray doses in the ranges of 25-100 r and 100-500 r. In each case the logarithm of the thrombin clotting time increased linearly with increasing dosage. The clotting delay due to irradiation was dependent on the thrombin concentration but independent of fibrinogen concentration. Addition, prior to irradiation, of sulfhydryl reagents failed to abolish the radiation-induced clotting defect. Irradiation of fibrinogen increased the rate of solution in concentrated urea of the fibrin clots obtained after addition of thrombin.

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Steroid Secretion by the Isolated Rat Adrenal.* (22363)

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(With technical assistance of Loma Coday.)

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Adrenal vein blood obtained from the rat has been demonstrated to contain several steroid hormones. Corticosterone is present in largest amount but hydrocortisone and aldosterone can also be detected(1,2). Bush found that the ratio of corticosterone to hydrocor-

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tisone secretion by the rat adrenal was approximately 20:1, whereas, in most other animals the secretion of hydrocortisone is greater than that of corticosterone(1). It has been demonstrated also that the rat adrenal gland secretes steroid hormones under *in vitro* conditions when stimulated by ACTH(3). Heard found that the rat adrenal incubated in the presence of ACTH secretes four steroids(4). On the basis of chromatographic mobility, ultraviolet light absorption, and staining reactions, these have been identified as corticosterone, cortisone, hydrocortisone, and an unknown substance.

The purpose of the present investigation was to determine the ratio of hydrocortisone and cortisone secretion to total steroid hormone production of the rat adrenal under *in vitro* conditions. To accomplish this, a method has been developed which permits quantitative determination of steroids containing an alpha-beta, unsaturated ketone structure in ring A and, also, steroids possessing a 17, 21 dihydroxy-20-ketone side chain. Thus, it is possible to determine the total steroid production of the isolated adrenal as well as the secretion of steroids such as hydrocortisone and cortisone. The results demonstrate that the secretion of hydrocortisone, cortisone, and other steroids possessing a dihydroxyketone side chain amounts to less than one-half of the total *in vitro* steroid production of the rat adrenal gland.

Procedure. Male Sprague-Dawley rats weighing 150-175 g were used in these studies. Animals were sacrificed by decapitation. The adrenals were quickly removed, dissected free of surrounding fat, weighed, and bisected. Each adrenal was placed in a beaker containing 2 ml of Krebs-Ringer-phosphate-glucose solution at pH 7.4. The glucose concentration in the medium was 200 mg%. One unit of ACTH was added to the contents of the beaker and the mixture incubated aerobically for 2 hours at 37°C in a Dubnoff metabolic incubator. On completion of incubation, the medium was decanted into a stoppered centrifuge tube leaving the adrenal slices in the beaker. The beaker was washed twice with one ml portions of distilled water and the rinsing water combined with the incubation medium in the centrifuge tube. The aqueous solution was extracted with 5 ml of ethylene dichloride and the mixture centrifuged at 1500 r.p.m. for 5 minutes. Following centrifugation, the aqueous phase was removed by aspiration and 4 ml of the ethylene dichloride extract was pipetted into a clean tube. The latter solution was taken to dryness under nitrogen at 50°C and the residue containing the steroids dissolved in 3 ml of methyl alcohol. Aliquots of the methyl alcohol solution were utilized for determination of the steroid concentration by a spectrophotometric technic and by the Porter-Silber method(5). Spectrophotometric measurement was accomplished by determining the absorption spectrum of the methanol solution from 220 to 260 mμ on a Beckman Model DU spectrophotometer. Steroids which possess an alpha-beta, unsaturated ketone structure in ring A demonstrate maximum absorption at 240 mμ. Steroid concentration was calculated from the absorption spectrum by the method of Allen (6). The Porter-Silber procedure measures only those steroids which have a 17,21 dihydroxy-20-ketone side chain such as cortisone, hydrocortisone, and Compound S.

Paper chromatographic separation of the steroid compounds secreted was achieved by combining the methyl alcohol solution obtained from 20 adrenal incubations. The toluene-propylene glycol chromatographic system of Zaffaroni was used(7). Twenty micrograms of compounds F, E and B were also placed on the chromatogram in order to compare the chromatographic mobility of the steroids secreted by the rat adrenal with that of known compounds. Steroids present on the chromatograms were detected by visualization under ultraviolet light and by staining with alkaline silver nitrate(7). Identification of the steroid secreted in largest quantity (steroid 4) was accomplished after elution of this substance from the chromatogram by use of the sulfuric acid chromogen technic(8).

Results. Recovery of steroids utilizing the method described was determined by adding known amounts of cortisone (free alcohol) to the incubation mixture. In these studies of

TABLE I. Recovery of Cortisone from Incubation Medium.

	Steroid recovery	
	Spectrophotometric method	Porter-Silber method
10.3 γ cortisone added	10.78 ± .19 γ* (104.7%) 11†	9.6 ± .11 γ* (93.2%) 12†

* Mean ± S.E. of mean.
† No. of determinations.

steroid recovery the adrenal slices were boiled to inactivate enzyme systems before being placed in the incubation medium. Table I demonstrates that recovery of cortisone was practically complete as measured by the spectrophotometric and Porter-Silber methods.

Table II compares the steroid secretion by isolated adrenals as measured by the spectrophotometric and phenylhydrazine-sulfuric acid methods. It is apparent that the spectrophotometric assay detects more than twice the quantity of steroid material determined by the Porter-Silber reaction.

TABLE II. Steroid Secretion by the Rat Adrenal Gland *In Vitro*; 19 Determinations in Each Series.

	Spectrophotometric method	Porter-Silber method
Actual steroid secretion/adrenal	11.4 ± 0.77 γ*	4.86 ± 0.24 γ
Steroid secretion per 100 mg adrenal wt	80.1 ± 5.28 γ	34.1 ± 4.58 γ

* Mean ± S.E. of mean.

The results of paper chromatographic separation of steroids secreted by rat adrenals are presented in Fig. 1. Four steroids are detected when the chromatogram is visualized under ultraviolet light and these are labelled in order of increasing mobility in the toluene-propylene glycol system. Steroid 1 has the same chromatographic mobility as does hydrocortisone. The mobility of steroid 2 is equal to that of cortisone. The third steroid band moves more rapidly than cortisone but slower than corticosterone. Steroid 4 moves at the same rate as does corticosterone in the toluene-propylene glycol system. Steroids 1, 2 and 4 reduce alkaline silver nitrate and it is, therefore, concluded that these steroids possess an alpha-ketol side chain.

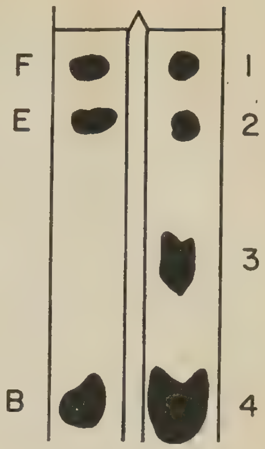

FIG. 1. Paper chromatographic separation of steroids secreted by isolated rat adrenal gland. Dark areas represent steroids which absorb ultraviolet light. Compounds F, E, and B were placed on left limb of chromatogram. The methanol solution containing the steroids secreted by 20 rat adrenals incubated with ACTH was placed on right limb of chromatogram.

Absorption spectra of the sulfuric acid chromogens of steroid 4 and that of corticosterone are presented in Fig. 2. It is apparent that the two spectral curves do not coincide. That of steroid 4 is, however, identical to the spectrum that has been reported for 11-epicorticosterone (Δ 4-pregnene-11 α, 21 diol-3, 20 dione) (9).

Discussion. The investigations of Bush(1)

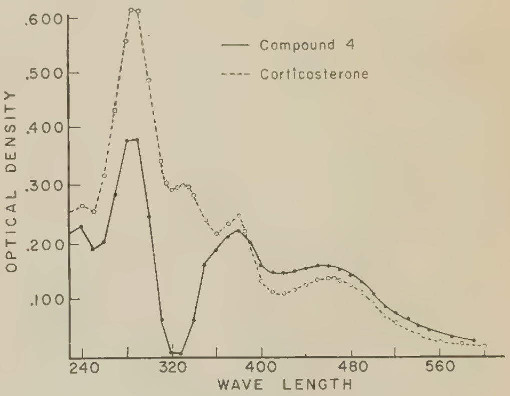

FIG. 2. Sulfuric acid chromogen absorption spectra of compound 4 and corticosterone. These steroids were eluted from the paper chromatogram, taken to dryness, and dissolved in concentrated sulfuric acid. Absorption maxima of compound 4 agree closely with those reported for 11-epi-corticosterone(9).

and Singer(2) have shown that adrenal vein blood obtained from the rat contains several steroid hormones including corticosterone, aldosterone, hydrocortisone, 11-dehydrocorticosterone, and 11-hydroxyandrostenedione. The steroid secreted in greatest quantity is corticosterone and the other hormones are produced in small amounts. Heard(4) has stated that the isolated rat adrenal cortex secretes both hydrocortisone and cortisone as well as corticosterone and an unknown substance. The results obtained in the studies reported herein also show that four steroids containing the alpha-beta, unsaturated ketone structure are secreted by the isolated rat adrenal. Quantitative measurement of steroid secretion demonstrates that almost one-half of the total adrenal hormones secreted under *in vitro* conditions consists of steroids which possess the 17, 21 dihydroxyketone side chain. The secretion of hydrocortisone, however, must account for only a small portion of the Porter-Silber reactive material since the area of the chromatogram containing this steroid was relatively small. If compound 2 is cortisone, as suggested by Heard, this would account for an additional quantity of steroid containing the dihydroxyketone side chain. The secretion of cortisone, however, was also small as judged by the area of the chromatogram containing this steroid. It is, therefore, possible that at least a portion of the steroid material present in area 3 contains a 17, 21 dihydroxy-20-ketone side chain. Heard(4) has obtained evidence which indicates that there are at least 2 steroids present in area 3 and believes that one of these is Compound S, a steroid which has a dihydroxyketone side chain and would, therefore, be measured by the Porter-Silber technic.

It has been demonstrated that corticosterone is the major hormone secreted by the rat adrenal *in vivo*(1). The steroid secreted in greatest quantity in these experiments possessed a chromatographic mobility equal to that of corticosterone. In addition, this compound, like corticosterone, contains an alpha-beta, unsaturated ketone in ring A and has an alpha-ketol side chain. However, the product formed by addition of sulfuric acid to this substance demonstrated an absorption spectrum different from corticosterone but identical to that reported for 11-epi-corticosterone. It is, therefore, concluded that under *in vitro* conditions the chief hormone secreted by the rat adrenal is 11-epi-corticosterone.

The explanation of the finding that the isolated rat adrenal secretes 11-epi-corticosterone, while under *in vivo* conditions corticosterone is produced, is not apparent. Previous investigations, however, have shown that there is a discrepancy between *in vivo* and *in vitro* enzymatic reactions concerned with steroid hormone metabolism. Dorfman (10) has demonstrated that if C_{21}-Δ^4-3 ketosteroids are incubated with liver or adrenal tissue, the reduction products formed are in the 5-alpha series. When these compounds are administered to the living animal, the reduction products obtained have the 5-beta configuration. Since in these investigations it has been demonstrated that an epimer of corticosterone is secreted by the isolated rat adrenal cortex, it is suggested that some of the other hormones produced *in vitro* may also be stereoisomers of the compounds secreted *in vivo*.

Summary. (1) A method to determine the *in vitro* steroid secretion by the rat adrenal is described. This procedure measures total steroid hormone production as well as secretion of steroids which have a 17, 21 dihydroxy-20-ketone side chain. (2) It has been demonstrated that the *in vitro* secretion of adrenal hormones which possess a dihydroxyketone side chain amounts to almost one-half of the total steroid secretion. (3) The isolated rat adrenal secretes four steroid hormones in response to ACTH. The compound secreted in largest amount has been shown to be 11-epi-corticosterone.

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Iodine Metabolism in a Region of Endemic Goiter. (22364)

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The great avidity for iodine exhibited by the thyroid gland of subjects living in certain regions where endemic goiter is prevalent has been demonstrated by Stanbury *et al.*(1,2) and by Roche *et al.*(3). It appeared to us of interest to carry out further measurements on different aspects of iodine metabolism in the regions previously studied by us. Results of thyroid and renal radioiodine clearance, conversion ratios, protein-bound I^{131}, stable iodine urinary excretion, rate of hormonal iodine formation and salivary radioactivity are reported in the present article.

Material and methods. All subjects were apparently healthy and euthyroid adults. One group (cases 1 to 14) came from the region of Bailadores, where previous studies had been carried out(3), and the other group (cases 15 to 21) from the region of Tabay, near the city of Mérida. Both regions lie within the Venezuelan Andes, at an altitude above 4,500 feet. For the study of thyroid I^{131} uptake, subjects were chosen whose thyroid was either barely palpable, or palpable but not readily visible. Radioiodine was supplied by Abbott Laboratories at Oak Ridge. External thyroid measurements were carried out with a Nuclear (Model DS-1) scintillation counter, placed 40 cm away from the thyroid cartilage. The resulting activity was multiplied by 0.85(1), and the activity measured 40 cm from the thigh was subtracted from the resulting value. The result was expressed as % of the administered dose, as measured 40 cm away from a 250 cc beaker (when the dose was given orally) or in a 20 cc syringe (when the dose was given intravenously). The beaker was thoroughly washed and the washings given to the patients. The syringe was counted before and after intravenous administration of the dose, and the second count was subtracted from the first. Measurements of plasma, saliva and urine radioactivity were performed in a well-type scintillation counter (Nuclear, Model 3037 B) and compared with the activity of suitably prepared standards.

Measurements of thyroid and renal clearances were carried out as follows: 50 μc of I^{131} in 10 cc of saline solution were injected intravenously. The thyroid was counted at 2 and 29 minutes during 2 consecutive minutes. The results, properly corrected, were taken to represent neck activity at 3 and 30 minutes respectively, and were expressed in terms of % of the administered dose. Blood was obtained at 5, 10 and 30 minutes and radioactivity of 3 cc of plasma was counted, compared with a standard and expressed in terms of % of the administered dose per liter of plasma. The patient was asked to urinate at 35 minutes and urine radioactivity was measured and compared with the standard. Thyroid clearance was calculated from a formula given by Kretchmar(4), modified from the method of Berson *et al.*(5):

$$T_c = \frac{N_{30} - \left[\dfrac{\dfrac{P_{30}}{P_3} \times N_3}{0.88}\right]}{\mu}$$

Where T_c = Thyroid clearance, N_{30} = Neck activity at 30 min., N_3 = Neck activity at 3 min., P_{30} = Plasma activity at 30 min., P_3 = Plasma activity at 3 min., calculated from curve constructed with the 5, 10 and 30 minute values and μ = Average plasma level, as measured planimetrically from the plasma radioactivity curve. The factor 0.88 was taken from Oddie(6). Renal clearance was calculated by dividing average plasma dose by urine concentration. Conversion ratio was established by counting radioactivity in 3 cc of plasma, precipitating with 6 cc 10% trichloracetic acid, washing twice with the same substance, and redissolving the precipitate in 3 cc 1 N NaOH. Saliva was obtained without stimulation, and the radioactivity was counted in the deep well scintillator. Urinary I^{127} concentration was determined chemically (7). The urines were collected in the usual way, their volume was measured and an aliquot was alkalinised, placed in glass-stoppered bottles, kept in the ice box and taken directly by plane within three weeks to the Boston Medical Laboratory where the determinations were performed. Rate of hormonal iodine secretion was calculated from the formula given by Stanbury *et al.*(2).

TABLE II. Conversion Ratios and Protein-Bound I^{131} in Subjects from Endemic Goiter Region.

Patient	Sex	Type of goiter*	Conversion ratio (%)	24 hr protein-bound I^{131} (% dose/L of plasma)
8	♂	D 1	96	.017
9	♂	D 2	97	.021
10	♂	N 2	100	.019
11	♂	D 2	42	.011
12	♀	N 2	52	.020
13	♂	D 2	90	.012
15	♂	D 2	51	.048
17	♀	N 3	75	.112
18	♀	N 3	90	.084
19	♀	N 3	64	.045
20	♀	D 1	87	.522
21	♂	N 3	87	.056
		Avg	78	.081
		Range	42–100	.011–.522

* N = Nodular; D = Diffuse. 1 = Barely palpable; 2 = Definitely enlarged, but not yet visible; 3 = Enlarged and visible.

Results. In Table I are shown results of thyroid uptake and clearance, renal 24 hr excretion and clearance of I^{131}, 24 hour I^{127} urinary excretion and daily rate of hormonal iodine secretion. All the patients in this table came from the region of Bailadores.

In Table II are listed the results of conversion ratios and 24 hour protein-bound I^{131}. In Table III we may see the results of sali-

TABLE I. Studies in Iodine Metabolism in Subjects from a Region of Endemic Goiter.

Patient No.	Type of goiter	Sex	24 hr thyroid I^{131} uptake (% of dose)	24 hr urinary I^{131} (% of dose)	Thyroid I^{131} clearance (ml/m)	Renal I^{131} clearance (ml/m)	Urinary I^{127} excretion (μg/d)	Rate of hormonal iodine secretion (μg/d)
1	No	♂	49.0	31.8	118.5	58.0	21.8	20.9
2	No	♂	54.8	25.9	124.4	43.7	19.8	24.0
3	N 2	♂	56.2	—	—	—	—	—
4	D 2	♂	52.0	33.2	82.6	35.7	9.6	10.4
5	D 1	♂	54.6	39.7	135.4	35.8	26.7	32.1
6	D 2	♂	55.3	15.3	130.5	42.7	19.8	24.5
7	D 2	♀	—	—	168.2	21.8	—	—
8	D 1	♂	43.2	13.2	86.2	51.5	21.1	16.0
9	D 3	♂	59.0	18.3	131.0	8.0	19.2	27.6
10	N 2	♂	53.0	22.7	110.3	18.8	20.0	22.6
11	D 2	♂	41.5	52.2	87.2	26.8	40.0	28.3
12	N 2	♀	36.1	79.5	38.5	35.3	35.4	20.0
13	D 2	♂	57.6	30.1	125.5	72.0	27.3	37.1
14	D 1	♂	43.1	12.5	70.7	32.0	9.0	6.8
Avg			50.4	31.2	108.4	37.1	22.5	22.5
Range			36.1–59.0	12.5–79.5	38.5–168.2	8.0–72.0	9.0–40.0	6.8–37.1

TABLE III. Saliva Radioactivity 24 Hr after Administration of 50 μc of I^{131}.

Patient No.	Saliva radioactivity (c/m/3 cc)
1	1262
2	713
3	1410
4	730
5	657
6	792
8	1412
9	727
10	961
11	1246
12	4670
13	654
14	2539
15	1604
16	640
17	2930
18	256
19	690
20	2819
21	91
Avg	1341
Range	91–4670

vary radioactivity measurements.

Discussion. The I^{131} studies are in accord with previous reports(2,3) and may be explained by a high iodine extracting efficiency of the thyroid. Thyroid clearance in these cases is generally well above the normal limits of 8-38 ml/m (mean 16) given by Myant *et al.*(8), of 26.3 ± 5.2 (standard error) given by Oddie *et al.*(6), and of 12.0-68.0 (mean 33.0 ml/m) given by Berson *et al.* (5). Although the 24 hour thyroid I^{131} uptake is not greatly elevated, and in some of the cases is even normal, a study of the thyroid clearance shows clearly that thyroid avidity for iodine is markedly increased in this region. In individuals with larger goiters, one would expect this avidity to be even higher. The only case who shows nearly normal values (case No. 12) was a woman who had lived for 15 years in Caracas, the capital, where thyroid uptake has been shown to be normal(9), and had lived in Bailadores for the last 3 years. Her diet was plentiful as compared to that of the other subjects in this study whose diet was almost exclusively of local vegetal origin(11).

Renal I^{131} excretion is somewhat reduced, but clearance is normal, showing that in these regions individuals do not develop the ability to conserve iodine through renal mechanisms. Normal renal iodine clearance is given by various authors as 11-44 ml/m, with a mean of 31 ml/m(8), 31.0-76.0 ml/m with mean 53.9(5) and 33.3 ± 3.1 (standard error)(10). Stanbury *et al.*, in Mendoza, in 3 subjects, found renal clearance values of 25, 29 and 44 ml/m respectively, all within normal limits also(2).

In cases 6, 8 and 14, the very low I^{131} excretion is suspicious, since total recovery of I^{131} was low; loss of urine is probable. Individual urine volumes in these cases were 1,100, 1,320 and 900 cc.

The 24 hour stable iodine excretion is quite low, confirming further the probability that the finding of increased thyroid avidity for iodine in this region is conditioned by iodine deficiency in food and water. Compensation for this iodine deficiency appears to be adequate in the cases studied, as evidenced by the clinical status and by the normal, although low, daily hormonal iodine secretion. A similarly low I^{127} excretion (17 μg/day average) had been found previously by De Venanzi *et al.* in individuals from a region nearby (13).

Saliva radioactivity has not been previously reported in a region of endemic goiter. In our cases, it was generally low, and corresponded approximately to the values one would expect in hyperthyroid patients with similar thyroid uptakes(12).

Conversion ratios are unusually high, and there seems to be an increased quantity of protein-bound I^{131} at 24 hours in the plasma. Here again, the findings resemble those of hyperthyroidism and are compatible with the idea that in the endemic goiter found in this region, as in the Mendoza goiter, there is increased extraction of iodine by the thyroid, a faster rate of iodine turnover, with an adequate quantity of hormonal iodine being synthesized.

Summary. Studies carried out in the Venezuelan Andes show that in this region there is an increase in thyroid clearance of iodine, thyroid uptake, conversion ratios and 24 hour protein-bound I^{131}. Renal clearance

of I^{131} is normal and urinary and salivary I^{131} low. Urinary I^{127} is low. It is concluded that in this region there exists a state of iodine deficiency, leading to compensatory thyroid avidity for iodine. There is no compensatory renal conservation of iodine.

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